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Critical Reviews and Perspectives

From liquid-phase synthesis to chemical ligation: preparation of oligonucleotides and their backbone analogs in solution

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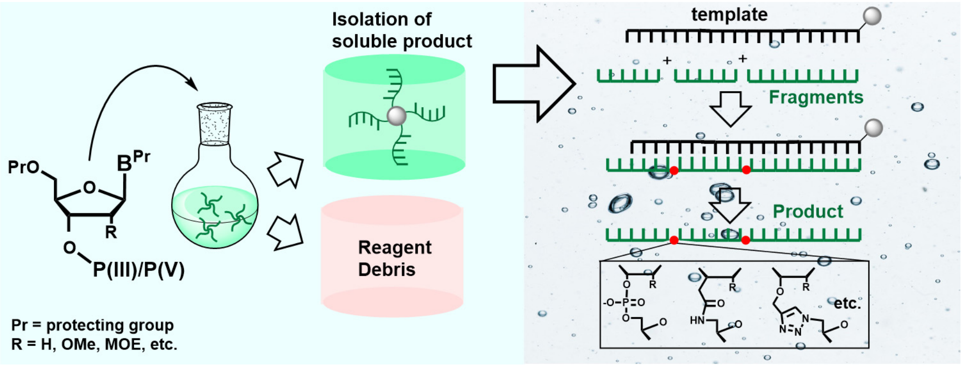
Abstract

The increasing need for sustainably produced oligonucleotides and their chemically modified counterparts has inspired numerous alternative scalable and greener production methods. Current manufacturing techniques depend on automated solid-phase synthesis and polymerase-based assembly, but liquid-phase synthesis and alternative engineered biocatalytic methods are emerging to meet the scale-up challenges. The scale, length, and modifications of the target nucleic acid product dictate the choice of protocol, which can be a combination of chemical and/or biocatalytic methods. While modern biocatalytic methods are competitive for the preparation of long oligonucleotides (up to 1 kb), liquid-phase synthesis is gaining a strong foothold for the large-scale preparation of short sequences (<30-mers), especially for the chemically modified therapeutic oligonucleotides. DNA-templated chemical ligation is an emerging technology expected to expand the range of liquid-phase synthesis to the production of longer sequences. This review focuses on oligonucleotide synthesis carried out exclusively in solution. Current protocols for liquid-phase synthesis of oligonucleotides and their backbone analogs, and template-assisted chemical ligation, yielding the phosphodiester linkage and its artificial mimics of biological relevance, are summarized.

Graphical abstract



Introduction

Sustainable manufacturing of synthetic nucleic acids of therapeutic applications is an emerging focus in the pharmaceutical industry [1–4]. More than 20 oligonucleotide drugs have reached the market for the treatment of a variety of disease targets [5]. In addition, they are increasingly valuable as diagnostic tools, DNA-encoded libraries [6], chiral catalysts [7], enzymes (i.e. DNAzymes), binders (i.e. aptamers) [8–10], and building-blocks of functional materials [11–13], including spatially defined nanostructures [14, 15], storage mediums of digital information [16, 17], and smart solar cells [18, 19]. The recent expansion of the use of nucleic acid analogs to large therapeutic market demand, e.g. cardiovascular diseases, hepatitis B infection, global viral pandemics, and cancers [5, 20], combined with their increasing value as versatile advanced biomaterials has challenged the current industrial manufacturing approach, which relies heavily on solid-phase oligonucleotide synthesis (SPOS) using phosphoramidite chemistry [21] and polymerase-based assembly employing nucleoside triphosphates as substrates [22, 23]. SPOS is a highly optimized [24], fully automated process that offers rapid and reproducible synthesis of chemically modified oligonucleotides on 1 mol scale. Because of the two-phase flow system, SPOS is not ideally suited for large-scale commercial manufacturing. Scalability and opportunities for improving sustainability for SPOS are limited. To ensure efficient chain

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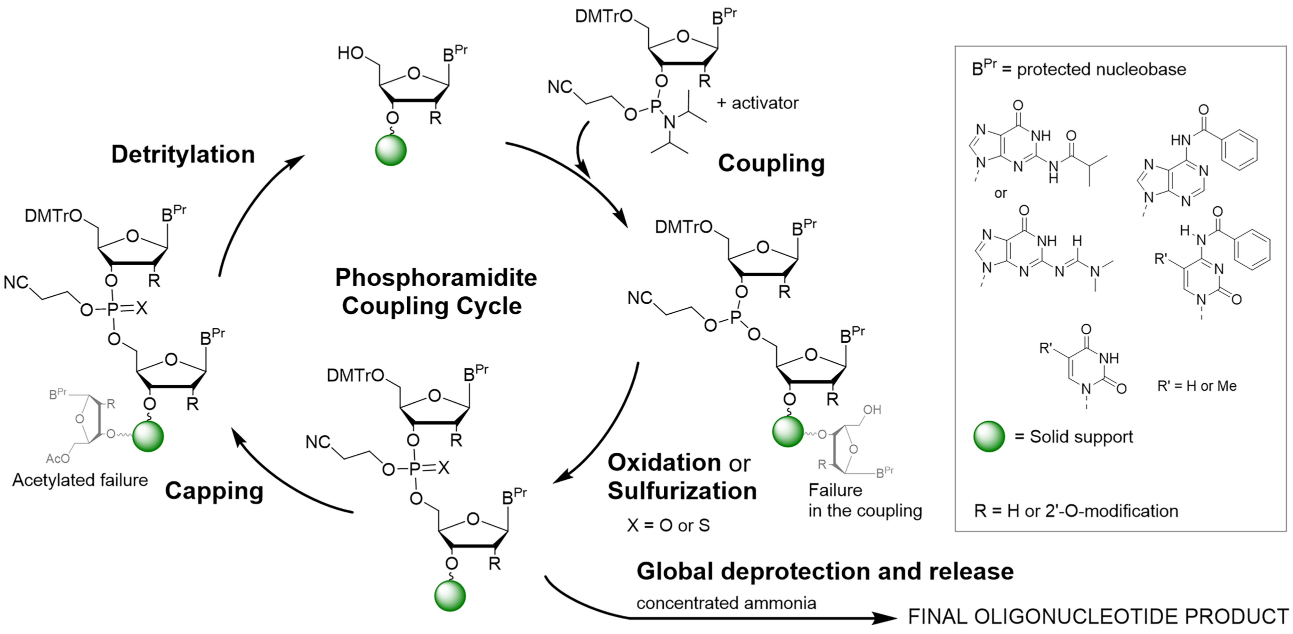


Figure 1. Schematic phosphoramidite coupling cycle in automated solid phase synthesis.

elongation in the solid phase, an excess of heavily protected nucleoside phosphoramidites and other reagents of the coupling cycle (Fig. 1) are needed to drive the reactions to completion, which compromises atom efficiency and produces byproducts that must be removed by extensive washing with organic solvents. These issues, together with chromatography-based downstream processing, results in a high process mass intensity (PMI) with a substantial waste stream, estimated to produce four tons of solvent and reagent waste per one kilogram of oligonucleotide (20-mer sequence) [1–4, 25]. Furthermore, synthesis of oligonucleotides longer than 100-mer is challenging using SPOS. Despite the high coupling efficiency of a single nucleotide (>99%), yields remain modest and marked analytical issues occur in the purification and quality control of linearly assembled products. Polymerase-based production avoids organic solvent use, aligns better with the sustainability demands, and is amenable for the production of long nucleic acids, several thousand nucleotides in length, needed for example for messenger RNA (mRNA) vaccines [22, 23]. The utility of polymerase-based oligonucleotide synthesis is limited for incorporation of chemical modifications, particularly in the backbone at precise location [26, 27]. These modifications are often required to improve the nuclease stability, therapeutic efficacy, and bioavailability of the oligonucleotide therapeutics, or to increase the chemical diversity and functionality of aptamers, aptazymes, and advanced nucleic acid materials.

To overcome the scalability and sustainability issues in current SPOS protocol, liquid-phase oligonucleotide synthesis (LPOS) and alternative biocatalytic methods are advancing rapidly (cf. previous reviews [1, 3, 4, 28, 29]). The most advanced LPOS methods have been demonstrated to be competitive for large-scale preparation of therapeutically relevant oligonucleotides up to 20-mer in length [30–34]. LPOS technologies utilize soluble anchors, which facilitate isolation of the intermediate products from the reaction media by membrane filtration, precipitation, or liquid–liquid extraction (Fig. 2). Because chain elongation can be performed entirely in homogeneous solution, real-time in-process analysis can be implemented, which is essential for multi kilogram production. However, because of the operational complexity and solubility/reactivity challenges of the growing protected oligonucleotide sequence, step-wise chain elongation by LPOS is not efficient enough to produce longer oligonucleotides (>30-mer), which are needed as potential binders, sensors, and catalysts.

Significant progress has been achieved in the production of modified oligonucleotides (see previous reviews: [2, 4, 35, 36]) with the advent of alternative biocatalytic methods. These methods employ temporally 3′-*O* or nucleobase protected nucleoside triphosphates for controlled polymerase-based template-independent chain elongation [37–41]. By repeating enzymatic one-nucleotide-extension, it can achieve the assembly of DNA sequences of up to 1 kb [42]. Further improvement of this technology aims at expanding the method to the synthesis of RNA oligonucleotides and chemically modified nucleic acid analogs. This improvement would further benefit from the use of more compatible temporary 3′-protecting groups of nucleotides and especially of ribonucleotides [43] or/and from further enzyme engineering to make this system more suitable for the synthesis of modified nucleic acids. The use of trinucleotide triphosphates, which allows enzymatic three-by-three-elongation of oligonucleotides, may provide a partial solution for by-passing substrate restriction of chemically modified units [44]. The efficiency and sustainability advantages of the biocatalytic template-independent approaches are obvious, but it may be noted that the methods reported to date rely on the use of solid support. A recent promising biocatalytic technology uses a DNA polymerase from *Thermococcus kodakarensis* to elongate a catalytic self-priming hairpin template with modified nucleotides, which is followed by endonuclease-mediated release of the product from the template [45]. This approach allows the use of a diverse array of modified building-blocks, including phosphorothioates and common 2′-modifications [2′-methoxy, 2′-*O*-methoxyethyl (MOE), 2′-fluoro, and locked nucleic acids]

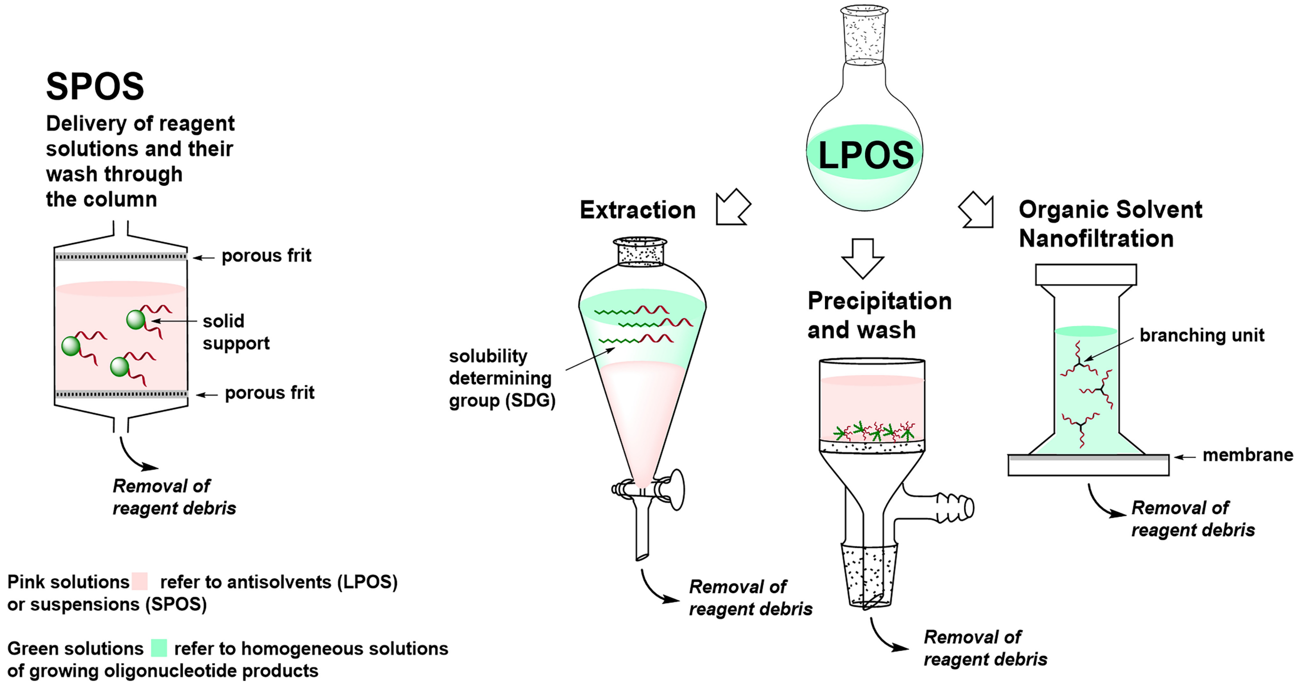


Figure 2. Comparison of isolation and purification of growing oligonucleotide products in solid-phase and liquid-phase oligonucleotide synthesis (SPOS versus LPOS).

(Fig. 3). The incorporation of phosphorothioate modifications in oligonucleotides results in all-*R*p stereoisomer products, but further enzyme engineering could expand this approach to the synthesis of products containing *S*p-stereoisomers [46]. Ligase-assisted methods have also undergone improvements. Ligases are rather promiscuous in terms of accepting modified substrates [47–55]. Their potential to form phosphodiester bonds between the 3′-OH and the 5′-*O*-phosphate group of fragments has been shown to be suitable for industrial-scale preparation of chemically modified oligonucleotides, including therapeutically relevant antisense oligonucleotides [56] and small interfering RNAs (siRNAs) [57, 58]. It is noteworthy that short 5′-*O*-monophosphate fragments (e.g. pentamers), readily accessible to LPOS, can be used as substrates by ligases.

The length, modifications, and scale of production of an oligonucleotide determine which synthesis method is best suited for its synthesis, which may in some instances require a combination of chemical and biocatalytic methods. Biotechnological approaches have shown high potential, especially for the manufacture of long oligonucleotides, but their competitiveness for the large-scale preparation of medium-sized chemically modified nucleic acids (<30-mer) requires catching-up with chemical synthesis. For immediate need, SPOS will likely remain the method of choice for the manufacturing of marketed backbone-modified oligonucleotide analogs, or at least fragments of these, and for recurring large-scale need, the same products can likely be produced by optimized LPOS. Precise control of the chirality of the phosphorothioate backbone in oligonucleotides is yet another area which is poised to grow in demand for these therapeutics [59, 60]. In this regard, recent progress in phosphorus chemistry at the P(V) oxidation level has advanced the efficient stereo-controlled synthesis of phosphorothioate oligonucleotides [61, 62], and due to its operational simplicity, it is suitable for LPOS [63, 64]. Three FDA approved oligonucleotide therapeutics are phosphorodiamidate morpholino oligonucleotides (PMOs) with an unnatural backbone structure [5]. After an uphill struggle, peptide nucleic acids (PNAs) [65] are also close to making a breakthrough as therapeutics [66–68]. Furthermore, because of their uncharged structure, unique binding characteristics, and high stability, they can be used in applications requiring advanced nucleic acid-based materials. Therefore, synthesis methods for process-scale manufacturing of PMOs and PNAs, which cannot be produced enzymatically, require additional efforts.

This review article does not provide an in-depth appraisal of the greenness [1] of different oligonucleotide production methods but primarily focuses on the protocols used for the chemical synthesis of oligonucleotides and their analogs executed in solution. LPOS of short to medium-sized oligonucleotides and oligonucleotide analogs (PMO and PNA) is summarized, including a discussion about the protecting group scheme, coupling chemistry, and solubility/reactivity challenges of the growing oligonucleotides in solution (cf. previous reviews regarding LPOS: 1, 3, 4, 28, and 29). Synthesis of fragments suitable for ligase-assisted assembly is a logical next step for LPOS. Therefore, promising examples of ligase-assisted synthesis deserve attention. In addition, ligase-assisted assembly acts as a benchmark for chemical ligation, which is discussed in more detail. Chemical ligation has been successful in producing a biologically active gene in the early 1990s [69]. It is somewhat surprising that it is yet to be commercialized in oligonucleotide production. Recent progress in DNA-templated chemical phosphodiester formation [70, 71] could pave the path for the chemical synthesis of longer (up to 100-mers) oligonucleotides in solution. Additional examples of the templated assembly of nucleic acid chains contain artificial backbone linkages. Specific examples are amide bond formation, reductive amination, and click-reaction in

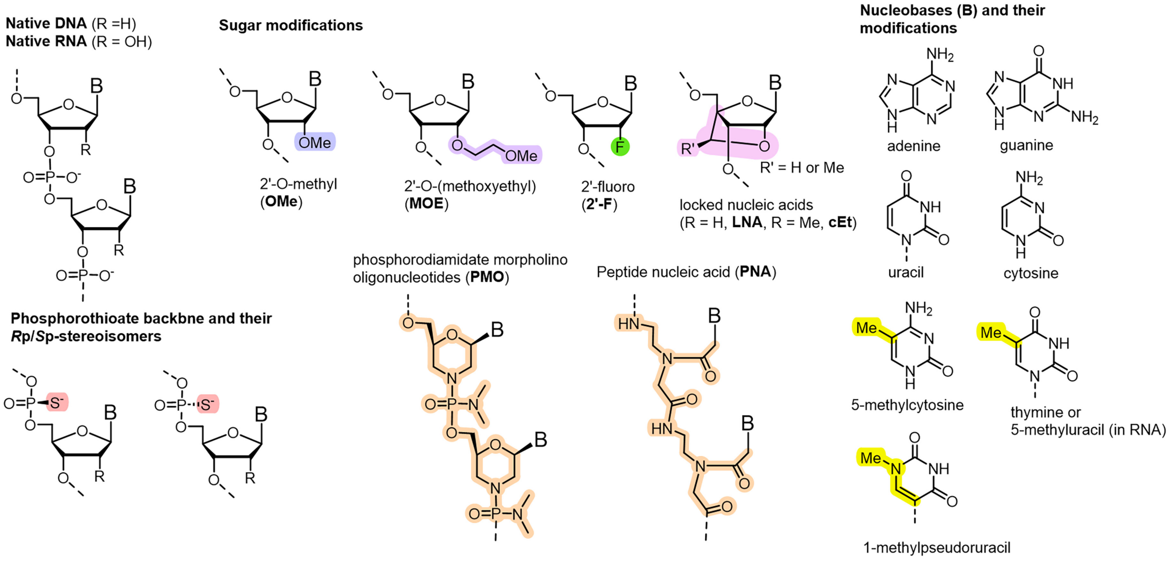
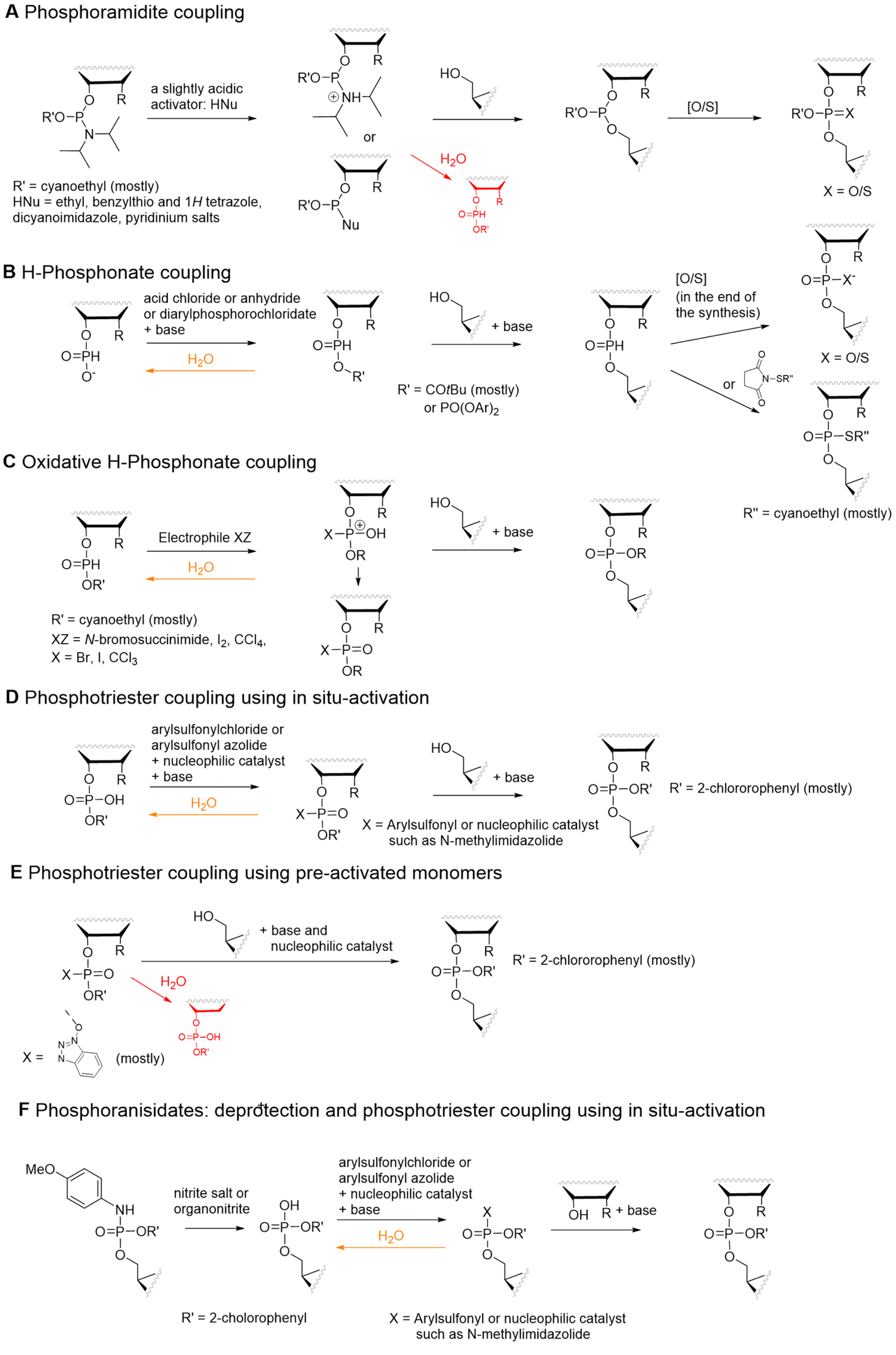


Figure 3. Common modifications and backbone analogs of oligonucleotides.

aqueous media. The incorporation of these modifications was translated into encouraging biological data which is noteworthy [72–75]. Chemical ligations, resulting in artificial backbone linkages, may also have potential for the discovery of functional molecules. Splitting aptamers and DNAzymes into smaller fragments, connected via an appropriate ligation reaction, is leading to the rapid development of advanced biosensors and offers the possibility of screening and producing nucleic acid analogs that cannot be synthesized via conventional enzymatic route (cf. previous review [76]).

Solid- versus solution-phase synthesis—pros and cons

Automated SPOS utilizing the phosphoramidite coupling cycle [21], introduced by Beaucage and Caruthers, has been foundational in the production of oligonucleotides for >40 years (Fig. 1). It employs 5′-*O*-(4,4′-dimethoxytrityl (DMTr))-protected nucleoside-3′-*O*-phosphoramidites as building blocks and consists of four steps: (i) detritylation, (ii) coupling, (iii) oxidation or sulfurization of the phosphite triester intermediate, and (iv) capping of the unreacted OH groups. After each step, reagent debris is removed by a simple wash with organic solvents, while the growing oligonucleotide is immobilized on a solid support (Fig. 2). This makes the process operationally simple and fully amenable to automation. Marked improvements in sustainability have also been achieved by reducing amidite/solvent consumption, finding greener and safer reagent/solution options, and recycling of the excess reagents [1, 24]. Despite these advantages, automated SPOS has encountered several scale-up issues. First, the heterogeneous reaction environment leads to nonlinear reaction kinetics, unequal distribution of reagents, and hindered access to the reagents for steric reasons, or just mass-transfer resistance between solution and solid phase [77]. Second, the solid phase material acts as a dead volume in the reaction suspension. Therefore, to achieve a complete reaction, more reagents for oligonucleotide synthesis are needed in the solid phase than are needed in a purely homogeneous solution. Third, real-time monitoring of solid phase reactions is limited, which complicates the optimization and fine-tuning of the reactions. A homogeneous reaction solution allows real-time-monitoring and thereby, controlled addition of reagents to minimize waste. Clearly, there are obvious advantages for producing oligonucleotides entirely in solution, but the isolation and purification of the growing intermediate products combined with the complexity of the practical implementation of the synthesis are major challenges. The optimal protecting group and coupling chemistry (Fig. 4) used for reactions in solution may need to be customized compared to automated SPOS (Fig. 1). High yielding convergent syntheses of oligonucleotides, including PMOs [78], and several other modalities in solution have been reported over the years [79–87]. Reese and Yan reported a convergent millimole-scale synthesis of Vitravene in solution using *H*-phosphonate chemistry (Fig. 4B) (Fig. 5) [83]. The *H*-phosphonate building blocks (1.2–1.4 equiv.) in the presence of diphenyl phosphorochloridate and *N*-(cyanoethylsulfanyl)succinimide as a condensation reagent and a sulfur-transfer reagent, respectively, were used to obtain protected trinucleotides with 88%–91% overall yields. The 5′-*O*-DMTr-protected trimers were converted to *H*-phosphonates, and the obtained trinucleotide blockmers (1.3–1.8 equiv.) were used for the assembly of a fully protected 21-mer Vitravene with 50% overall yield. Global deprotection resulted in the final product. In this type of convergent synthesis, representing a classical solution-phase synthesis, each intermediate product was isolated by column chromatography (extractive work ups were sufficient for some synthesis steps of trimers, Fig. 5) and characterized in detail. While this synthesis is high yielding and reagent efficient (cf. the overall consumption of the building blocks in Fig. 5), the chromatographic steps are far less practical and more time consuming than the solvent washes used for SPOS. In the following sections, various LPOS methods are described, in which simple workups only, i.e. extraction, precipitation, and organic solvent nanofiltration (OSN) (Fig. 2), are used upon chain elongation of oligonucleotides. Soluble anchors, defined as soluble supports, solubility determining groups (SDGs), and branching units, are utilized to facilitate



For simplicity, partial nucleoside structures around the phosphate linkage are depicted. R = H or 2'-O-modification

Figure 4. Various coupling chemistries used for oligonucleotide synthesis during LPOS.

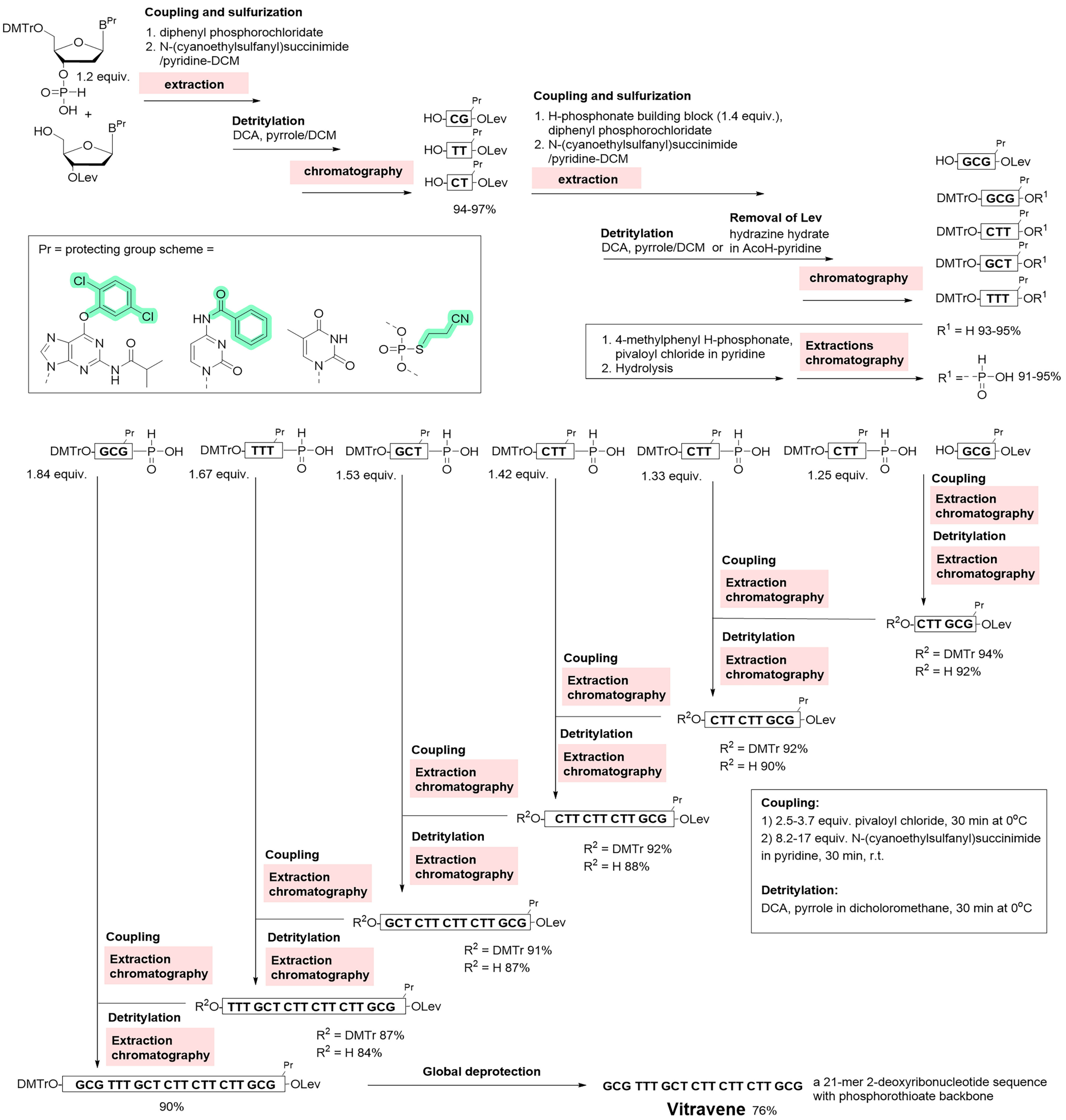
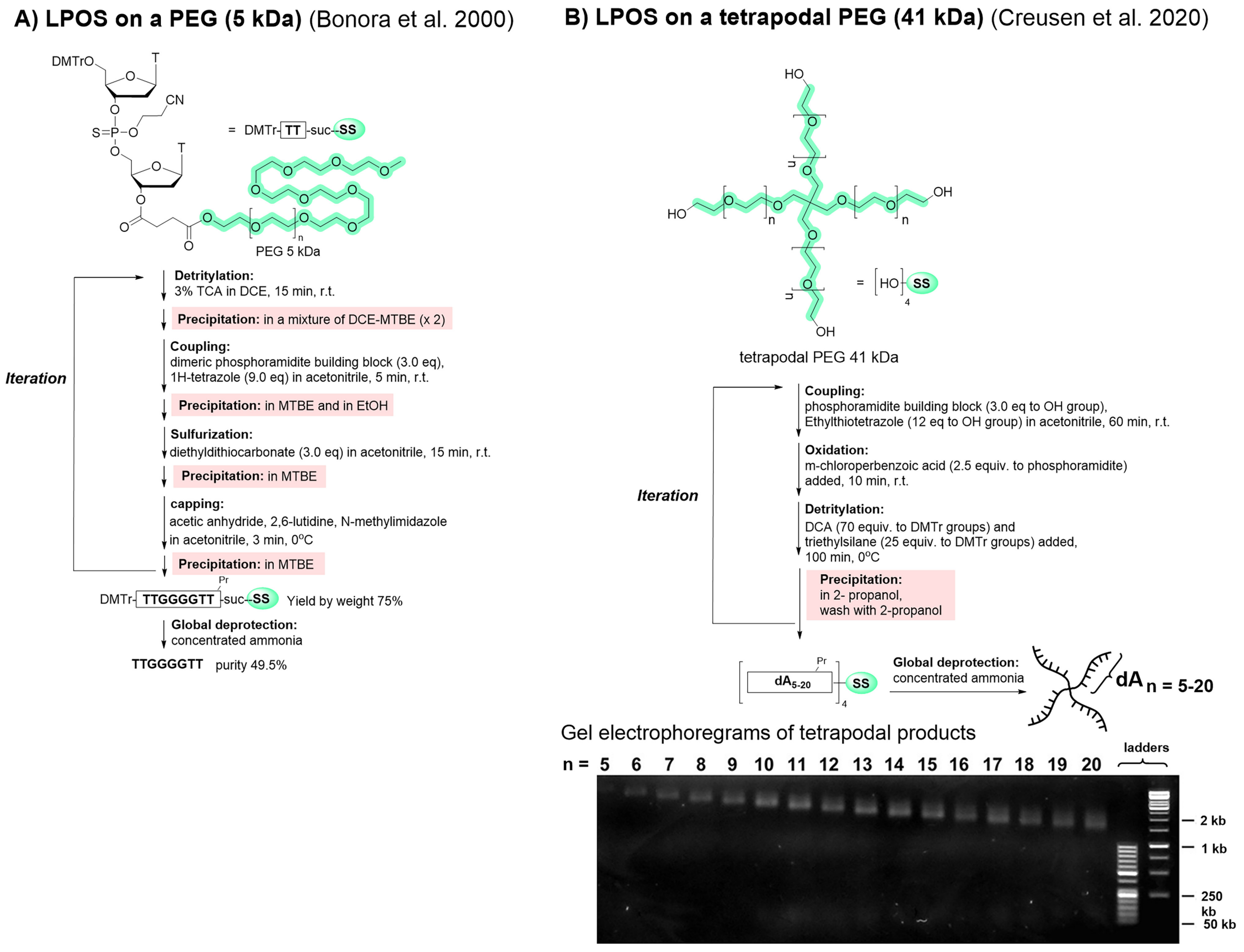


Figure 5. Classical solution-phase convergent synthesis of Vitravene (Reese and Yan, 2002).

the isolation and purification of the growing oligonucleotide by altering its physicochemical properties (often increased hydrophobicity).

Liquid-phase oligonucleotide synthesis on soluble supports

Fully protected oligonucleotides, consisting of cleavable protective groups on their 5′- and/or 3′-OH groups, exocyclic amino groups of nucleobases, and a phosphate backbone, can be lipophilic enough to be precipitated in polar organic solvents or/and washed during aqueous extraction to remove impurities. As seen in the convergent synthesis above, the growing oligonucleotide products of variable lengths can be washed by aqueous solutions (Fig. 5). In an ideal scenario, the reactants used for chemical chain elongation and their traces can be removed via precipitation from a solvent or water washing [88]. However, as the chain lengthens, the solubility of the protected oligonucleotide may change depending on its sequences and the nature of the protecting group utilized. This has led to the development of soluble supports or SDGs to improve isolation and purification of the intermediate products. This concept is not new. Alongside early steps in solid phase synthesis reported by Merrifield [89, 90], the idea of applying soluble supports to the synthesis of organic biopolymers also emerged [91]. Even in



Pr refers to standard protecting group scheme, including nucleobase and cyanoethyl protections

Figure 6. Liquid-phase synthesis on PEG soluble supports. **6B** adapted with permission from {Creusen, G., Oluwadunsin, C., Schumann, K., Walther, A. (2020) Scalable One-Pot-Liquid-Phase Oligonucleotide Synthesis for Model Network Hydrogels. *J. Am. Chem. Soc.*, **142**, 16610–16621.}. Copyright {2025} American Chemical Society.

the 1960s, the advantages of soluble supports versus solid supports were evident under homogeneous reaction conditions offering better accessibility of reactants and linear reaction kinetics [91–93]. The seminal works by Cramer [94], Khorana [95, 96] and coworkers in the late 1960s reported the use of soluble polystyrene (molecular mass: 270 kDa) for the synthesis of short thymidine trimers in solution. The first thymidine was bound to the support via its 5′-O-protecting group (monomethoxytrityl) and synthesis proceeded in the 5′→3′-direction. The growing oligonucleotide on the soluble polystyrene support could be isolated from the reactant debris by precipitation in water. At the same time, cellulose [97], polyethylene glycol [98], and polyvinyl ether [99, 100] were proposed as potential soluble supports for liquid-phase synthesis. However, the success of chain elongation on early soluble supports was limited by the coupling chemistry of the time (a condensation of nucleotide 5′-monophosphates). Using more advanced phosphotriester chemistry (Fig. 4D) [101], Kamaike *et al.* assembled an 11-mer ribonucleotide and an 8-mer deoxyribonucleotide on cellulose acetate [102]. Coupling with 5′-O-DMTr-protected tri- or tetranucleotide-3′-(*o*-chlorophenyl)phosphates in the presence of triisopropylbenezene sulfonyl chloride and *N*-methylimidazole in pyridine, acetic anhydride capping, and detritylation by an acid-catalyzed methanolysis in dichloromethane were performed in one vessel, and the cellulose acetate-bound product was precipitated in ethanol. Bonora *et al.* introduced high efficiency liquid-phase (HELP) oligonucleotide synthesis using polyethylene glycols (PEG 5kDa and PEG 10 kDa) as soluble supports [103]. The growing protected oligonucleotides on the PEG supports can be isolated by precipitation in ethers (Et_2O or methyl-*tert*-butyl ether, MTBE). Different chain elongation methods employing phosphoramidite [104], phosphotriester [105], and H-phosphonate chemistries [106] have been used with PEG supports (Fig. 4). The applicability of HELP has been demonstrated for millimol-scale synthesis of 20-mer phosphorothioate oligonucleotides [107]. Synthesis of an 8-mer 2′-deoxyribonucleotide by HELP is shown in Fig. 6A. Dimeric phosphoramidite building blocks (3 equiv. in each coupling) were used to reduce the overall number of precipitations. The protected PEG-supported product was obtained with a crude yield of 75%. Global deprotection with concentrated ammonia resulted in a final oligonucleotide product with 50% purity according to IE-HPLC analysis. Borona's

PEG strategy was a promising approach for large-scale production, but the number of precipitations required (six per synthesis cycle) made this technique less desirable. It is worth noting that no sperate oxidation step is required in phosphotriester chemistry (Fig. 4E) [105] simplifying the coupling cycle and reducing the number of precipitations. Padiya and Salunkhe synthesized a 10-mer deoxyribonucleotide on a PEG (5 kDa) support by using oxidative coupling of cyanoethyl *H*-phosphonate building blocks [106] (Fig. 4C). The couplings with the alkyl *H*-phosphonates (2.5 equiv. to OH group) were performed in a mixture of triethylamine and acetonitrile using *N*-bromosuccinimide as an activator (5 min at room temperature), followed by precipitation in Et_2O and re-precipitation in Et_2O-acetonitrile mixture. The unreacted 5′-OH groups were capped using a mixture of acetic anhydride and *N*-methylimidazole in lutidine, and the growing product was precipitated by Et_2O. DMTr group was removed by 6% dichloroacetic acid (DCA) in dichloromethane (15 min), followed by precipitation in Et_2O and re-precipitation in Et_2O-DCA mixture. Iteration of this synthesis cycle followed by global deprotection with concentrated ammonia resulted in a 10-mer 2′-deoxyribonucleotide with a yield of 75%. The purity of the oligonucleotide produced by this method was not appreciably different from that of the same oligonucleotide produced by SPOS.

More recently Walther and coworkers used a tetrapodal PEG of 41 kDa for LPOS [108]. Branched oligonucleotides, 20-mer homo thymidine (T_{20}), and homo 2′-deoxyradenosine sequences (dA_{20}), permanently attached to the tetrapodal PEG, were synthesized on a multigram scale and used as constituents for self-assembled supramolecular network hydrogels. Synthesis of the tetrapodal homo 2′-deoxyradenosine construct is shown in Fig. 6B. The reaction steps of the nucleoside extension cycle were performed in one vessel, followed by only one precipitation in 2-propanol at −30°C. A mixture of 2′-deoxyadenosine-3′-*O*-phosphoramidite building block (3 equiv.) and ethylthiotetrazole in anhydrous acetonitrile, followed by oxidation with *m*-chloroperbenzoic acid, was used for each coupling. A relatively large excess of dichloroacetic acid (70 equiv. to DMTr groups) and triethylsilane (25 equiv. to DMTr groups) as a trityl scavenger was added to the coupling cocktail at 0°C. After a detritylation time of 100 min, the product was precipitated in 2-propanol at −30°C, washed with the same solvent, and dried. Then the synthesis cycle was repeated. The average recovery yield per step throughout the synthesis was 94%. Because of the uniform and radially symmetric soluble support-oligonucleotide construct, the reaction steps could be monitored by ^1H and ^{31}P NMR, and the homogeneity of the deprotected products after each nucleotide extension could be verified by gel electrophoresis (Fig. 6B). Despite the 2′-deoxyadenosine-rich sequence and harsh detritylation conditions, no depurination was observed. Recently, tetrapodal PEGs (5, 10, 20, and 40 kDa) have also been used as soluble supports in OSN-based LPOS (discussed below) [109].

Designer polymeric soluble supports have gained popularity for solution-phase synthesis of peptides, including different PEG variants, fluorous tags, and more advanced hydrophobic polymers (cf. previous review [111]). However, such organic polymers have yet to be tried for LPOS. One reason may be the decreased activity of reactive groups on stiff and large molecular weight polymers, making reaction efficiency on these supports not appreciably different from those on solid supports [77]. Thus, the initially sought benefits of liquid-phase synthesis may not be forthcoming. It should be noted that 5, 10, 20, and 40 kDa PEGs were used in the forgoing LPOS techniques. These PEGs represent macromolecular soluble supports rather than polydisperse polymers.

Liquid-phase oligonucleotide synthesis using solubility determining groups

Preliminary trials with an adjusted protecting group scheme, cyclodextrin anchors, and ionic liquids

Instead of polymeric soluble supports, lipophilic SDGs (<2 kDa) and branching units have attracted more interest in recent LPOS techniques. Van der Marel and coworkers proposed adjusting the protecting group scheme to facilitate extractive workups in LPOS [111]. A lipophilic 3′-*O*-adamantyl acetate tag (**I**/Fig. 7) of the first nucleoside and N^3-pivaloyloxymethyl (Pom) protection of thymidine residues were used to increase the lipophilicity of the oligonucleotides [112]. After each coupling and oxidation, the intermediate product was dissolved in EtOAc and the reaction debris removed by aqueous extraction. DMTr was deprotected by a HCl/MeOH cocktail and the released DMTrOMe was removed by acetonitrile–hexane extraction. Standard 5′-*O*-DMTr-protected phosphoramidite building blocks (and Pom-protected dT-building blocks, 1.5 equiv. per coupling) were used for the synthesis of a hexamer deoxynucleotide, with a 39% overall yield after HPLC purification. Lönnberg and coworkers used acetylated and methylated *β*-cyclodextrin as solubility tags (**II**/Fig. 7) [113]. Coupling was achieved using 5′-*O*-(2-methoxypropyl, MIP)-protected phosphoramidite building blocks (1.5 equiv. to OH group) in the presence 4,5-dicyanioimidazole in a mixture of acetonitrile and dichloromethane, followed by oxidation with wet iodine in pyridine and DCM-$NaHSO_3$ extraction. MIP was removed by a mild HCl treatment in methanol-dioxane, and volatiles were removed *in vacuo*. The use of MIP-protected nucleosides for synthesis resulted in easy removal of the protecting group fragments, acetone and methanol. In some cases, the dextrin-bound product remained contaminated by reagent residue, and flash chromatography was required for further purification. Iteration of this synthesis cycle, followed by global deprotection with concentrated ammonia and HPLC purification, gave a pentamer 2′-deoxyribonucleotide in a 52% yield. Damha and coworkers introduced a novel approach by attaching the first nucleoside to an imidazolium tetrafluoroborate (**III**/Fig. 7) [114]. The imidazolium tetrafluoroborate tag formed a soluble ionic liquid-based support. 5′-*O*-DMTr-protected phosphoramidite building blocks were used for chain elongation and the growing product was isolated by precipitation (twice after coupling and twice after detritylation in EtOAc-Et_2O) and aqueous extraction after oxidation. Using this method, di-, tri-, and tetranucleotides were synthesized in high yield and purity.

These protocols based on the SDG concept show potential for further development. They could have been improved by employing alternative technical implementation (reducing the number of extractions and evaporation, and whether flash chromatography was needed) and by slightly modifying the structures of the SDGs. For example, the solubility properties of *β*-cyclodextrin can be adjusted by adding other *O*-alkyl groups (R in Fig. 7).

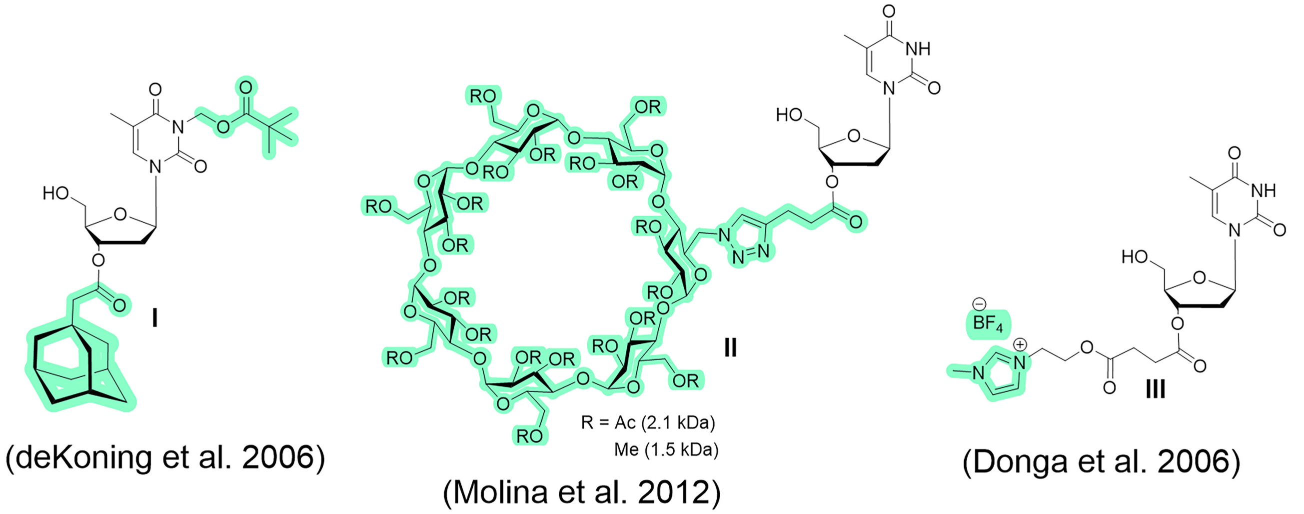


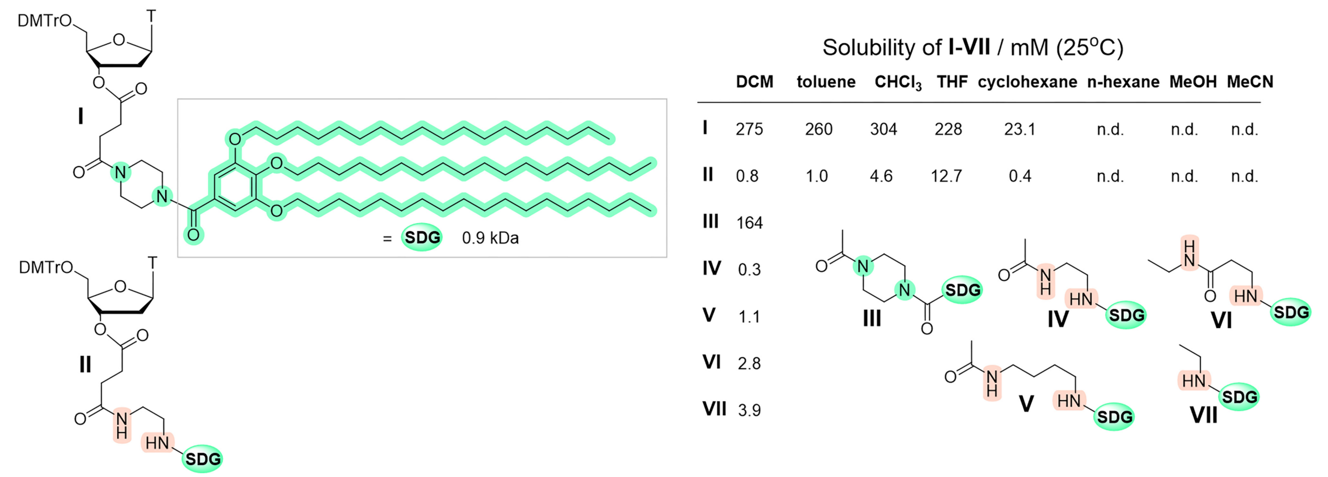
Figure 7. Structures of adamantyl acetate (**I**), clyclodextrins (**II**), and imidazolium ion (**III**) solubility tags used for LPOS.

Applications of long aliphatic alkyl chains as solubility determining groups

Tamiaki [115] and Chiba *et al.* [116] introduced lipophilic SDGs of long aliphatic alkyl chains for solution-phase synthesis of peptides. Protected peptides with these SDGs could be isolated by precipitation in polar organic solvents (acetonitrile and alcohols) or by extraction, including a thermomorphic extraction [116]. These SDGs were also found to be appropriate for LPOS of RNA (Fig. 8) [117]. To identify optimal SDGs for LPOS, Chiba *et al.* studied the solubility of gallic acid-derived SDGs, which consist of different spacer structures, a succinyl-linked nucleoside (**I** versus **II** / Fig. 8) and their variants (**III** versus **IV–VII**/Fig. 8), in different solvents [117]. Primary amides (**II**/Fig. 8) on the spacer structures had a negative effect on the solubility of these SDGs in aprotic organic solvents, resulting in hydrogen-bonded aggregates of the soluble support constructs. Gallic acid-derived SDG comprising a piperazine-succinyl linker (**I**/Fig. 8) was used for LPOS of a 21-mer RNA with a TT overhang. Each coupling was performed by exposing 2 equiv. of standard phosphoramidite building blocks to benzylthiotetrazole, followed by oxidation with 2-butanone peroxide in dichloromethane. A mixture of DCA in dichloromethane was used for detritylation. Growing intermediate products were isolated from the reaction byproducts by precipitation in methanol. Two precipitations per synthesis cycle were used: first after coupling and the second after detritylation. The fully protected RNA was obtained with a yield of 46%. Global deprotection with concentrated ammonia and triethylamine trifluoride released the desired RNA in 78% purity. A 4-carboxybenzyloxycarbonate and a 2-oxyacetate variant of **I**/Fig. 8 have also been used for the liquid-phase synthesis of protected tri- and tetranucleotides [118, 119], which are useful precursors for convergent assembly. The protected oligonucleotides were orthogonally released from the soluble support by mild reducing conditions.

Ajinomoto Co. Inc. and BACHEM (https://www.bachem.com/articles/oligonucleotides/scalable-tag-assisted-one-pot-liquid-phase-oligo-synthesis/) have applied similar lipophilic SDGs for oligonucleotide synthesis and have developed the most advanced LPOS processes to date, namely *Ajiphase* and *Molecular hiving*, respectively. The strategy developed by Ajiphase was initially established for the large-scale synthesis of peptides [120], and later applied for LPOS [30, 31] and PMO synthesis [121]. A set of different SDGs, including galloyl-derived benzyl groups with different aliphatic alkyl chains, was evaluated to further optimize the solubility/precipitation/extraction/reactivity properties of the growing protected biooligomers (Fig. 9). There are fundamental differences between the methods used for peptide and oligonucleotide syntheses. Peptide synthesis is not as water sensitive as oligonucleotide synthesis, and it can operate in a stationary organic phase (chloroform used) which can be washed by aqueous extraction after each coupling and deprotection. No evaporation of the solvents or precipitation of the intermediate products is needed in this continuous extraction system, which reduces overall solvent and energy consumption, and makes the process operationally simple. The LPOS strategy of Ajiphase is based on the precipitation of intermediate products, which are dried prior to the next coupling cycle. The number of precipitations is reduced by performing detritylation, coupling, and oxidation in one vessel, in this order. Interestingly, the organic salts (i.e. pyridinium trifluoroacetate) formed in the neutralized detritylation cocktail can act as activators in the following coupling step. In this telescope process, only one precipitation in methanol per nucleotide extension cycle is required. Examples of the peptide and oligonucleotide synthesis by Ajiphase are shown in Fig. 9A and B. Commercially available building blocks, i.e. Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids and 5′-O-DMTr-protected nucleoside phosphoramidites, have been used for the assembly. A fully protected 20-mer peptide and a 20-mer oligonucleotide have been synthesized with crude yields of 79% and 60%, respectively. Global deprotection was performed using a TFA cocktail (peptide) and concentrated ammonia (oligonucleotide).

Biogen validated the applicability of lipophilic alkyl chain SDGs for the convergent LPOS of an 18-mer gapmer oligonucleotide drug candidate consisting of an 8-mer 2′-deoxynucleotide window, MOE ribonucleotide wings and a mixed phosphorothioate-phosphodiester backbone (Fig. 10) [32]. 5′-O-DMTr-protected tetra- and pentamer fragments (**I–IV**/Fig. 10) were synthesized in 0.3 and 3 kg scale batches using 3′-O-*tert*-butyldiphenylsilyl (TBDPS)-protected nucleosides as starting materials. The 3′-O-TBDPS and the stan-



Solubility of **I-VII** / mM (25°C)

	DCM	toluene	$CHCl_3$	THF	cyclohexane	n-hexane	MeOH	MeCN
I	275	260	304	228	23.1	n.d.	n.d.	n.d.
II	0.8	1.0	4.6	12.7	0.4	n.d.	n.d.	n.d.
III	164							
IV	0.3							
V	1.1							
VI	2.8							
VII	3.9							

Figure 8. The role of linker structures to the solubility of soluble anchors (Kim *et al.* 2013).

dard nucleobase protecting group scheme allowed intermediate products to be extracted and precipitated in heptane-TBME, resulting in fragment yields of 62%–76% and purities of 90%–95% [122]. The reactions steps (coupling, sulfurization/oxidation, and detritylation) were performed sequentially in the same vessel (telescoped) and only 1.05–1.4 equiv. of the nucleoside phosphoramidites were used for assembly. The 2′-MOE uridine with a 3′-*O*-gallic acid-derived alkylchain SDG was coupled to the 3′-fragment (**IV**→**V**) and the SDG was then utilized for the assembly of phosphitylated fragments, facilitating isolation of the intermediate products by precipitation in acetonitrile or heptane. Again, a telescoped approach was applied, but two precipitations/coupling cycle were required: first after coupling and the second after detritylation. For efficient ligation, 2–5 equiv. of the fragments was required. After treatment with concentrated ammonia, the 5′-DMTr-protected 18-mer product (**X**/Fig. 10) was obtained at a scale of 200 g with a yield of 49% and a purity of 80% without the need for chromatography. Critical impurities, such as those derived from unremoved phosphoramidite monomers, incomplete coupling, over-coupling, and incomplete detritylation, were monitored throughout synthesis. In comparison with SPOS, consumption of monomeric phosphoramidites and solvents was reduced by 47% and 28%, respectively. The same procedure was extended to the synthesis of a 34-mer oligonucleotide. It is notable that efficient synthesis of therapeutically relevant oligonucleotide end products could be achieved by using tetra- and pentanucleotide fragments as building blocks (i.e. blockmers). Alternative methods can be applied for the synthesis of these blockmers, including classical solution phase synthesis and LPOS [118, 119, 123–125]. Damha and coworkers introduced recently a mechanochemical synthesis of short DNA and RNA fragments [126–128]. The method operates without bulk solvent and it can be combined with solubility anchors. With a proper protecting group selection, it could also be applied for the sustainable blockmer synthesis.

Liquid-phase synthesis using branching units

A variety of tri- and tetrapodal branching units, such as pentaerytritol [129], tetrakis(4-hydroxyphenyl)methane [130, 131], adamantane [130, 132], and benzene-1,3,5-triol derivatives (with octaethylene glycol arms, homostar) [33, 34], have been utilized as "branched soluble supports" for LPOS (**I–IV**, Fig. 11). While these soluble supports may affect solubility in the beginning of the synthesis, they act as branching units rather than solubility anchors when the length of the oligonucleotides increases. In the end of the synthesis, the overall construct is consisted mainly of protected oligonucleotides, and its solubility is strongly dependent on the protecting groups utilized, discussed below (note the difference in comparison to tetrapodal PEGs described by Walther, Ferguson and coworkers [108, 109]). The branched oligonucleotide structures can be precipitated near-quantitatively in polar protic solvents to remove the reagent residue during LPOS. The other attractive feature of branched oligonucleotides is their potential rejection during OSN protocol [33, 34, 109].

Virta and coworkers introduced a pentaerytritol-derived tetra azide core unit for LPOS of short oligonucleotides (**I**/Fig. 11) [129]. The following factors are considered important in the design of the core: (i) straightforward synthesis of the scaffold, (ii) efficient attachment of the first nucleoside, equipped with a linker, (iii) a symmetric structure that facilitates HPLC and NMR characterization of the growing products, and (iv) the potential for purification of the branched products by OSN or precipitation in protic organic solvents (methanol or 2-propanol). With the pentaerytritol core, 2′-deoxyribonucleotides and ribonucleotides up to 5-mers have been synthesized by Lönnberg group [129, 133, 134]. Phosphoramidite [129] and phosphotriester chemistries [135], different protecting group schemes, and linker structures have been utilized. Disulfide [123], hydroquinone-*O,O*'-diacetic acid ("Q") [124, 136], and levulinic ester linker [125] have been used for the orthogonal cleavage of fully protected trinucleotide blockmers. Typically, two precipitations per nucleotide extension are used for the isolation of the growing products: first after 5′-*O*-deprotection and the second after coupling. LPOS of short stereo-defined phosphorothioate fragments on this core has also been reported [64]. Recently, Baran and coworkers developed limonene-derived oxathiaphospholane sulfide (Ψ) chemistry [61, 62], an extension of the Stec protocol [137–139], which was a substantial improvement for the synthesis of stereo-defined phosphorothioate oligonucleotides. With the P(V) chemistry, excellent efficiency and stereospecificity can

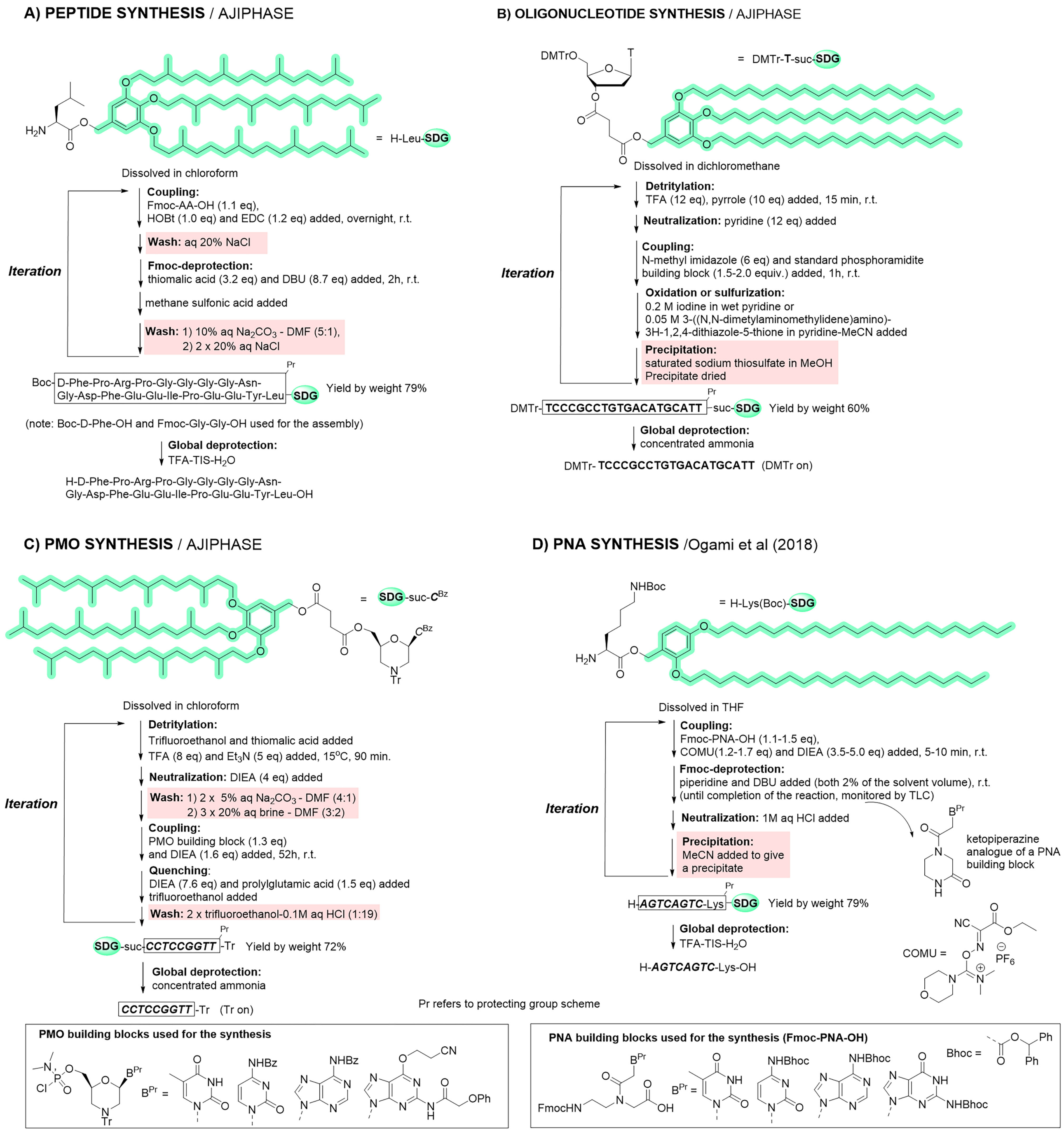


Figure 9. Liquid-phase synthesis of peptides (**A**), oligonucleotides (**B**), PMO (**C**), and PNA (**D**) using SDGs of long aliphatic alkyl groups. Note: Conditions can vary depending on the scale and sequence of the biooligomers assembled.

be achieved, and because of the redox-neutral coupling, operating under basic conditions, it is well suited for LPOS. Short stereo-defined phosphorothioate fragments have been assembled on the pentaerytritol core using 5′-O-(2-methoxypropyl) (MIP)-protected 2′-deoxynucleotides as Ψ-activated building blocks (Fig. 12) [64]. The couplings were performed with Ψ-building blocks (1.7 equiv. to OH group) in the presence of DBU (1,8-diazabicyclo[5,4,0]undec-7-ene, 11 equiv. to OH group) in a mixture of pyridine-MeCN (15–60 min), followed by neutralization with acetic acid and precipitation in 2-propanol. MIP was removed using a mixture of 4% DCA in dichloromethane-methanol (3–6 min), followed by neutralization with pyridine, and another precipitation in 2-propanol. By repeating this synthesis cycle, including two precipitations, and exposing the protected products to global deprotection at the end of synthesis, stereo-defined tetranucleotide fragments were obtained. The stereochemical purity of the products was verified by ^{31}P NMR. Products could be isolated with an average yield of 80% per synthesis cycle, leading to 53%–59% yields of tetranucleotides. In contrast to the other LPOS methods mentioned above, the exposed phosphorothioate backbone is elongated, which makes the growing sequence hydrophilic, reducing precipitation efficiency. However, the simple and efficient P(V) chemistry and

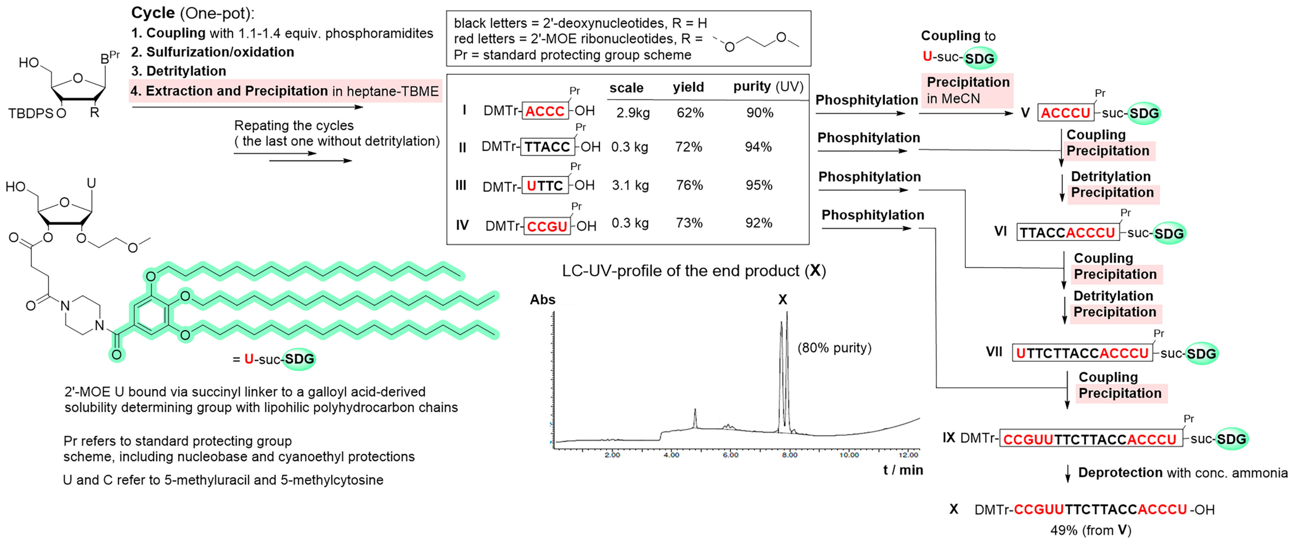


Figure 10. Schematic convergent synthesis using a solubility determining group. Adapted with permission from {Zhou, X., Kieasman, W. F., Yan, W., Jiang, H., Antia, F. D., Yang, J., Fillon, Y. A., Xiao, L., Shi, X. (2022) Development of Kilogram-Scale Convergent Liquid-Phase Synthesis of Oligonucleotides. *J. Org. Chem.*, **87**, 2087–2110} Copyright {2025} American Chemical Society.

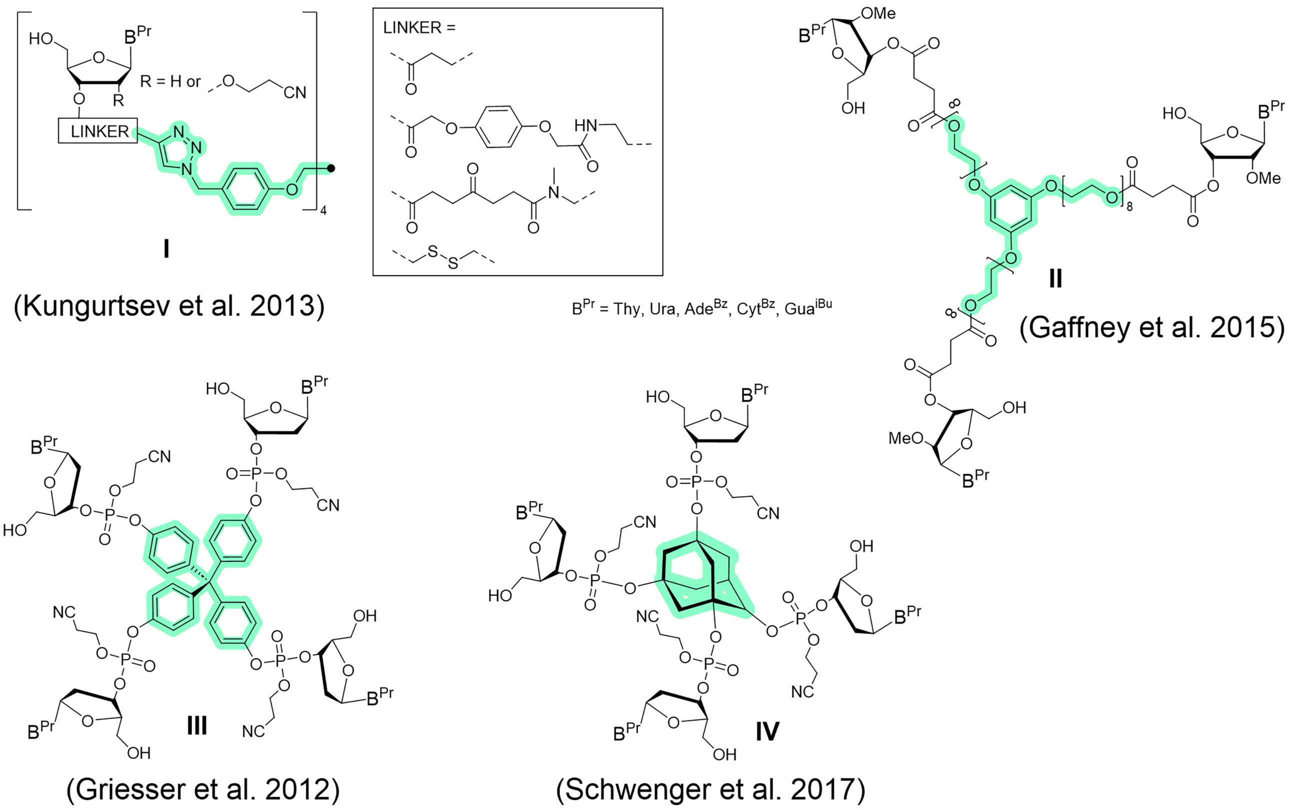


Figure 11. Examples of branching units used for LPOS.

easy 5′-O-MIP deprotection proved to be a valuable combination to LPOS approach. The precipitation efficiency could be improved by using alternative protecting group schemes, or other soluble support/work-up options. Overall, this preliminary work demonstrates the power of LPOS for the synthesis of stereo-controlled phosphorothioate fragments. Efficient ligation of such pre-characterized fragments with a 3′- or 5′-monophosphate group could be used for synthesis of therapeutically relevant products (discussed below).

Richert and coworkers synthesized branched oligonucleotides on an adamantane-1,3,5,7-tetraol core in solution (Fig. 13) and used them as constituents of self-assembled nucleic acid materials, based on hybridization-mediated networks (cf. nucleic acid hydrogels by Walther *et al.* [108])

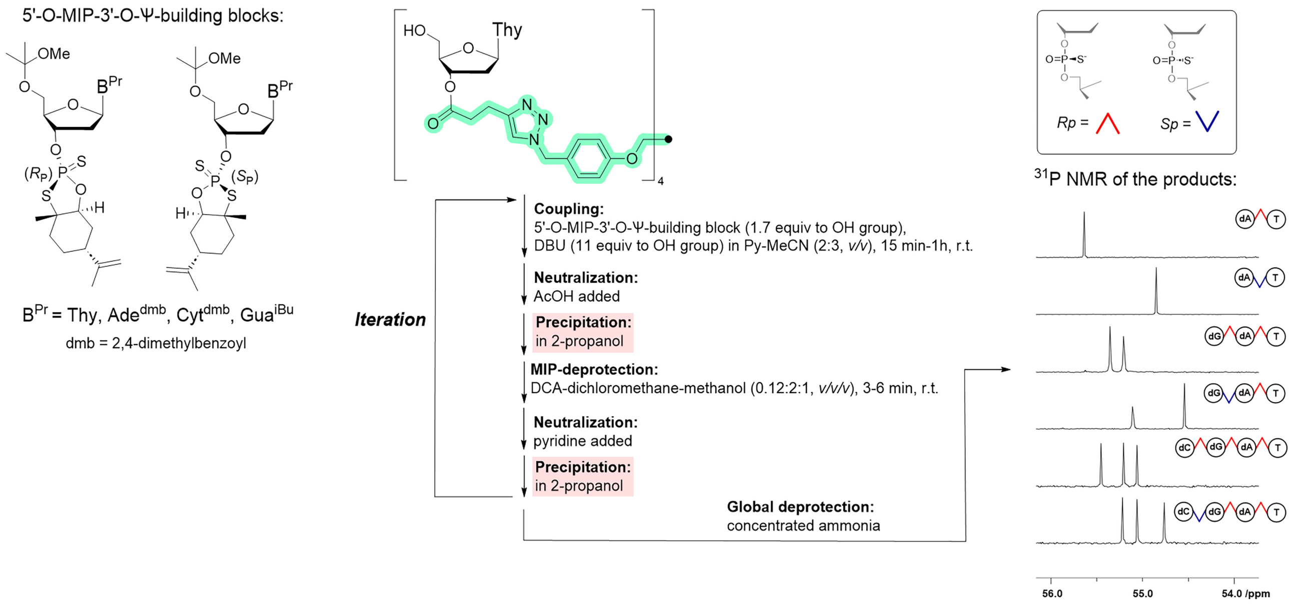


Figure 12. Stereo-controlled liquid-phase synthesis of short phosphorothioate oligonucleotides (Rosenqvist *et al.*, 2023).

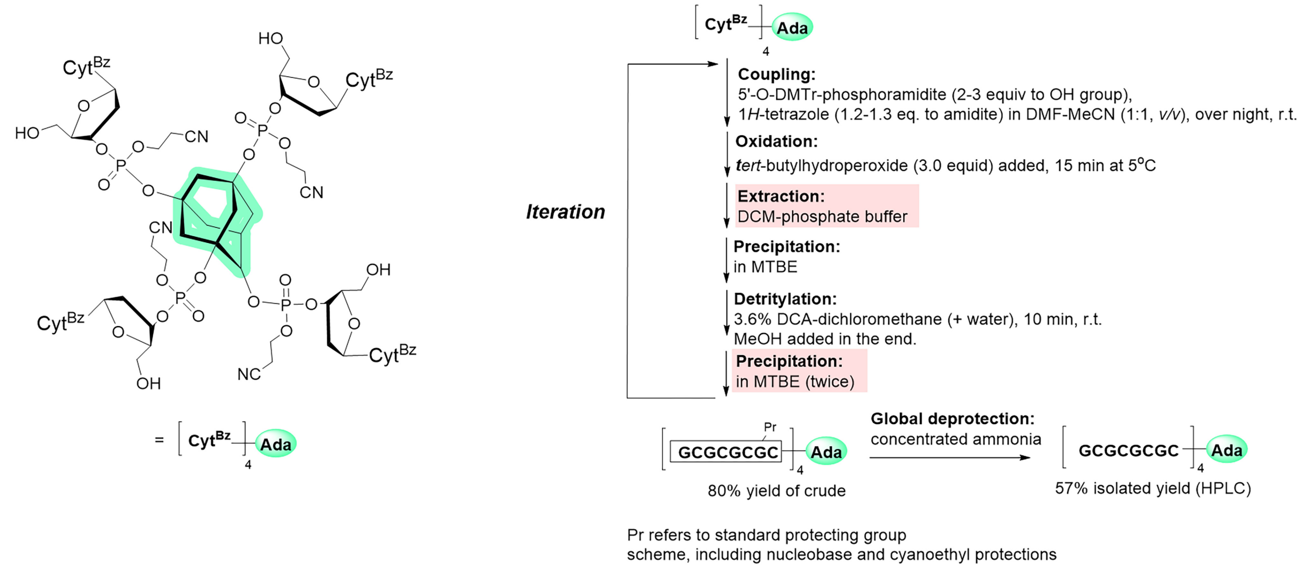
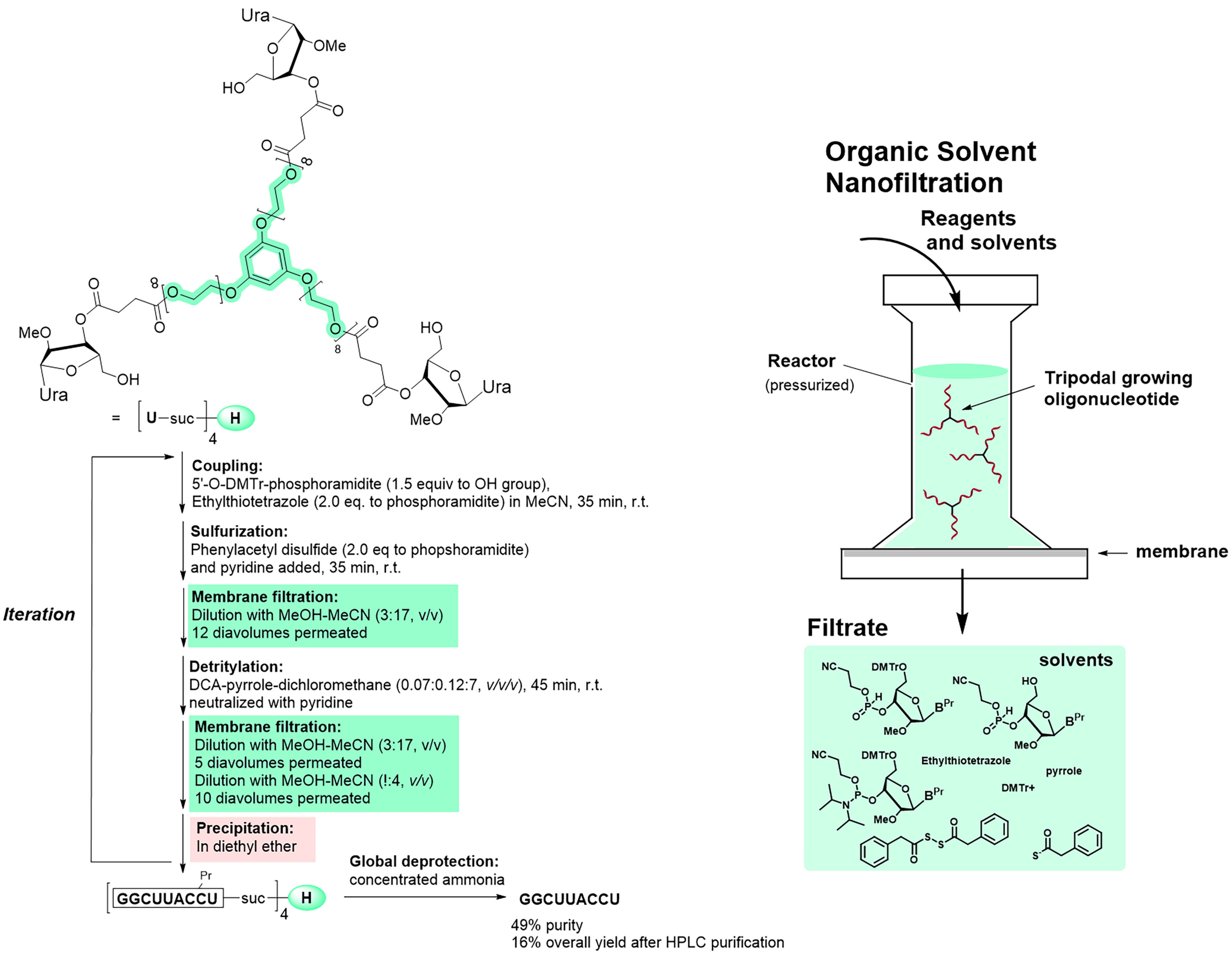


Figure 13. Liquid-phase synthesis of tetrapodal oligonucleotides on an adamantane core (Schwenger *et al.*, 2017).

[132]. The couplings were performed in a mixture *N,N*-dimethylformamide (DMF) and acetonitrile using standard 5′-*O*-DMTr-protected phosphoramidite building blocks (2 equiv. to OH group) and tetrazole (1.2–1.3 equiv. to the amidite) as an activator. *Tert*-Butyl hydroperoxide was added to the coupling cocktail to oxidize the phosphite triester intermediate, and then the mixture was diluted with dichlorometane. The reagent residue was removed by washing the mixture with an aqueous phosphate buffer, and the product was precipitated in MTBE. DMTr was removed with a mixture of 4% dichloroacetic acid (DCA) in wet dichloromethane and the product was precipitated twice in MTBE. By repeating this synthesis cycle, followed by final global deprotection, tetrapodal oligonucleotide products with up to 8-mer arms and variable sequences were efficiently assembled by LPOS. An example of tetrapodal 32-mer nucleotide synthesis is shown in Fig. 13.

Organic solvent nanofiltration

In the LPOS methods, extractions and precipitations are used to isolate the growing oligonucleotide and remove reagent debris. These methods are scalable and suitable for industrial production, as demonstrated by Ajinomoto and BACHEM, but concerns may arise about whether these methods are truly practical in metric ton scale, in which efficient recycling of solvents and reagents is required. Appropriate nanofiltration with membranes of precise molecular weight cut-offs can be utilized to facilitate solvent and reagent recycling. Bayer and Mutter demonstrated the use of membrane filtration for liquid-phase synthesis of peptides [91] and oligonucleotides [99], but the membranes at that time were unstable in organic solvents and only permitted modest molecular size discrimination. With more advanced polymeric membranes, described by Livingston *et al.*, OSN fulfilled the critical separation

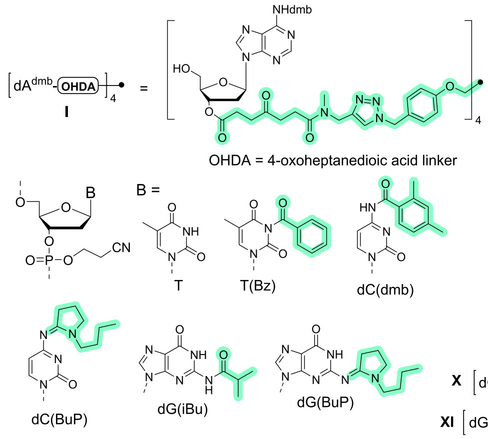


Pr refers to standard protecting group scheme, including nucleobase and cyanoethyl protections

Figure 14. Liquid-phase oligonucleotide synthesis using OSN (Gaffney *et al.*, 2015).

criteria for LPOS [33, 34]. In this system, the reactions and filtrations are done in a single reactor. The rejection and permeation properties of the solute determines which membranes and reagents can be used for OSN. The question is about diafiltration, which enriches target molecules in the rejected solution with continuous solvent refilling of the reactor. In early trials, oligonucleotides have been assembled on a tripodal homostar core (**II**/Fig. 11) and poly(benzimidazole)-membrane, cross-linked with para-dibromoxylene (PBI-17DBX), was used for nanofiltration. PBI-17DBX is resistant to chemical degradation during chemical chain elongation of oligonucleotides and shows good reproducible performance in acetonitrile. The tripodal branched structure inhibited threading of the oligonucleotide product during membrane permeation, leading to its rejection by the membrane, while small molecular reagent debris and solvents could be easily removed by filtration through the membrane. The radially symmetric structure facilitated real-time-monitoring of the reactions by HPLC and NMR. The applicability of OSN was demonstrated by gram scale LPOS of a 9-mer 2′-O-methyl ribonucleotide (Fig. 14). The couplings were performed in acetonitrile using 5′-O-DMTr-2′-O-methyl ribonucleotide-3′-O-phosphoramidites (1.5 equiv. to the OH group) and ethylthiotetrazole (ETT, 3.0 equiv. to the OH group) as an activator. Phenyl acetyl disulfide (PADS, 3 equiv. to the OH group) was used for sulfurization and the mixture was subjected to diafiltration. After 12 diavolumes, the retentate was evaporated and the residue was dissolved in a mixture of 1% DCA and pyrrole in DCM, neutralized by addition of pyridine, diluted with methanol and acetonitrile, and then nanofiltration with 10 diavolumes of a methanol–acetonitrile mixture was carried out. After filtration, the product was precipitated in diethyl ether to remove contaminating DMTr-pyrrole (and in some cases monomeric amidite traces) and dried. Iteration of this synthesis cycle, followed by a final global deprotection step with concentrated ammonia, gave a 9-mer 2′-O-methyl ribonucleotide product with a purity of 49%, and after HPLC purification, the overall yield was 16%. After initial validation, substantial improvements (e.g. membrane materials and practical implementation) in OSN-based LPOS have been achieved. Currently, development of OSN technology is pursued at Exactmer. It is worth noting that an advanced and automated OSN-based system is already available for the preparation of peptides [171]. Recently, Ferguson and coworkers used ceramic membranes (Inopor NF 750 and UF-2000 Da) for OSN-based LPOS [109]. A one pot approach, integrating sequential cou-

Table 1. Physical parameters (A) and solubility (B) of soluble support constructs on a pentaerytritol core (Rosenqvist *et al.,* 2024).



A

		Molecular mass /kDa	Protecting group content, including the branching unit /m%	Mass concentration used for LPOS* /g L^{-1}
I	[dA(dmb)-OHDA]$_4$•	3.0	67	36
II	[T-dA(dmb)-OHDA]$_4$•	4.5	50	53
III	[T(Bz)-dA(dmb)-OHDA]$_4$•	4.9	55	58
IV	[dC(dmb)-T-dA(dmb)-OHDA]$_4$•	6.3	47	75
V	[dC(BuP)-T-dA(dmb)-OHDA]$_4$•	6.3	47	75
VI	[dC(dmb)-T(Bz)-dA(dmb)-OHDA]$_4$•	6.8	50	80
VII	[dC(BuP)-T(Bz)-dA(dmb)-OHDA]$_4$•	6.7	50	80
VIII	[T-dC(dmb)-T-dA(dmb)-OHDA]$_4$•	7.8	41	92
IX	[T-dC(BuP)-T-dA(dmb)-OHDA]$_4$•	7.7	41	92
X	[dG(iBu)-T-dC(dmb)-T-dA(dmb-OHDA]$_4$•	9.6	39	114
XI	[dG(BuP)-T-dC(BuP)-T-dA(dmb)-OHDA]$_4$•	9.8	40	116

* corresponds to 0.012 mol L-1 solutions of **I-XI**

B

soluble support construct	MeCN	MeCN/DCM (1:1, *v/v*)	DCM	DCM-MeOH (1:1, *v/v*)	MeCN-DMF (1:1, *v/v*)	MeCN-Py (4:1, *v/v*)	MeOH	2-propanol	MTBE / g L^{-1}
I	0.12	29 ± 2	>300	>300	> 300	16 ± 2	0.5	≈ 0	≈ 0
II	0.007	1.1	0.5	>300	> 300	13 ± 1	0.06	≈ 0	≈ 0
III	1.2 ± 0.4	>300	>300	>300	> 300	>300	0.02	≈ 0	≈ 0
IV	0.004	0.8	0.3	>300	> 300	10 ± 1	≈ 0	≈ 0	≈ 0
V	4.4 ± 0.4	13 ± 1	0.11	>300	> 300	>300	3.0 ± 0.1	≈ 0	≈ 0
VI	26 ± 1	>300	>300	>300	> 300	>300	18 ± 1	≈ 0	≈ 0
VII	48 ± 2	>300	>300	>300	> 300	>300	32 ± 1	≈ 0	≈ 0
VIII	0.004	0.15	0.04	>300	> 300	7.0 ± 0.2	≈ 0	≈ 0	≈ 0
IX	1.7 ± 0.4	5.8 ± 0.9	0.06	>300	> 300	130 ± 20	5.0 ± 0.9	0.003	≈ 0
X	0.006	0.3	0.003	>300	> 300	3.0 ± 0.3	0.01	≈ 0	≈ 0
XI	0.5	2.3 ± 0.4	0.07	>300	> 300	14 ± 4	1.1 ± 0.4	0.003	≈ 0

Note: The solubility values <1 g l^{-1} and >300 g l^{-1} are given with one-digit precision without error limits. A solubility of 300 g l^{-1} represents an upper limit (a value clearly more than needed for synthetic purposes).

pling (1.5 equiv. phosphoramidite building blocks and 4 equiv. dicyanoimidazole, DCI, to the OH group, 60 min, r.t., followed by quenching with MeOH), sulfurization (3 equiv. 3-phenyl 1,2,4-dithiazoline-5-one, POS, to the OH group, 30–60 min, r.t.), and detritylation (a mixture of TFA in the presence of cyclohexanethiol scavenger in MeCN-DCM, 2:1, v/v, 30–130 min, 0°C, followed by neutralization with pyridine) was used. A tetrapodal PEG (20 kDa) was used as a soluble support and a single membrane purification using 5 diavolumes of MeCN-MeOH (9:1–4:1, v/v) per one nucleotide extension cycle by a house-made cross flow filtration device was employed. An 18-mer 2′-OMe phosphorothioate oligonucleotide was synthesized with 97%–100% stepwise filtration yields and a purity of 72%, which was a marked achievement. A sustainability analysis of the OSN–LPOS resulted in an approximated PMI of 718 kg/kg per nucleotide, but it could be decreased when a proper solvent recovery is integrated into the system. In comparison in the same calculation [109], the corresponding PMIs obtained for SPOS and precipitation-based LPOS described by Biogen (Fig. 10) [32] have been estimated to be 200 and 63 kg/kg, respectively. The green round table report [1] estimates similar PMI values (152–251 kg/kg per nucleotide) for SPOS of eight oligonucleotides in development.

Role of solubility in liquid-phase synthesis

The sizes of most branching units and SDGs used for LPOS are relatively small compared with those of longer protected oligonucleotides. For example, the convergent synthesis, described above (**V–IX**/Fig. 10), uses an SDG of 1 kDa, and the molecular weights of the protected pentamer, decamer, 14-mer oligonucleotide, and 18-mer oligonucleotide attached to it are 2.3, 4.5, 6.2, and 6.6 kDa, respectively. Thus, the physicochemical properties of the protected oligonucleotides themselves dominate as synthesis progresses, and the contribution of SDG to solubility decreases. This is more obvious when branching units are used for LPOS. These features are reminiscent of the swelling properties and suspension volumes of solid supports, which can differ dramatically between the beginning and end of biopolymer assembly [140, 141]. Horvath and Virta *et al.* examined the solubility of tetrapodal soluble support constructs (protected tetra-, octa-, hexadeca-, and eicosanucleotides) in different solvent systems (Table 1) [142]. In general, the solubility of protected oligonucleotides was marginal in the protic solvents methanol and 2-propanol and in ether (MTBE), convincingly demonstrating their potential as antisolvents in precipitation step (Table 1B). The observed solubility was also low in acetonitrile, dichloromethane, and in mixtures of acetonitrile–dichloromethane and acetonitrile–pyridine, which are common solvent systems in oligonucleotide synthesis (Fig. 15). The soluble support constructs were readily soluble in methanol–dichloromethane and dimethylformamide–acetonitrile mixtures. These two solvent systems have been used for 5′-*O*-deprotection and couplings using the pentaerytritol core [123–125, 129, 133–135, 142]. A significant portion of the mass of the protected oligonucleotide comes from

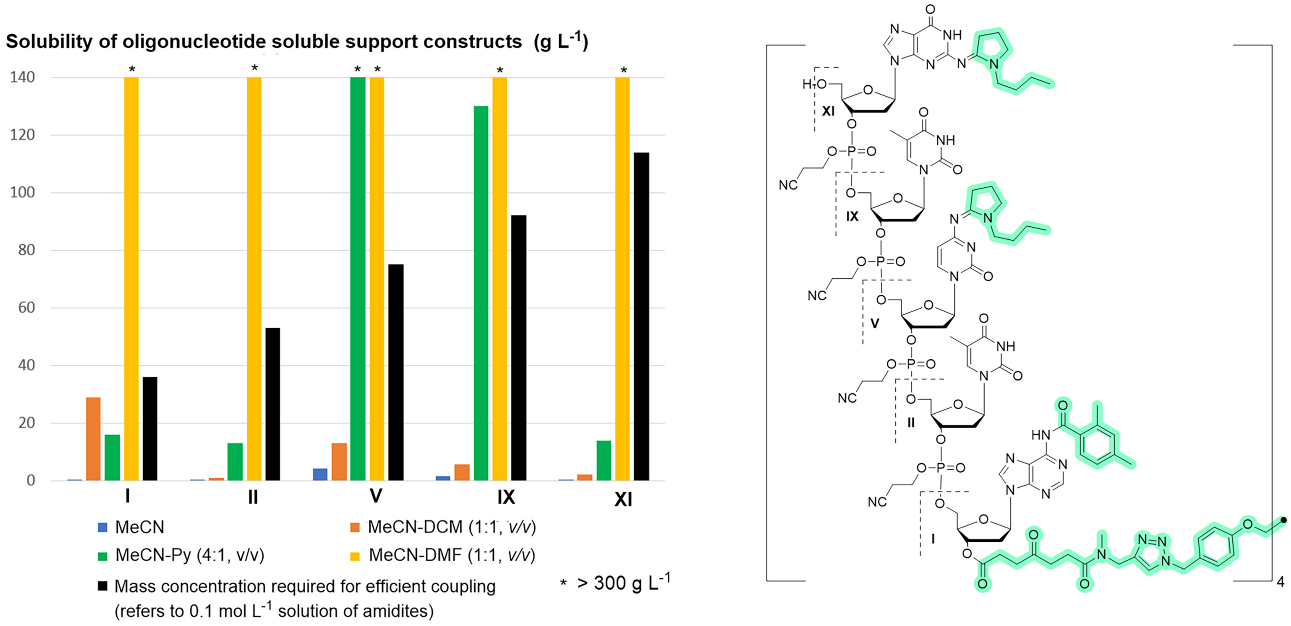


Figure 15. Solubility of tetrapodal oligonucleotide-soluble support constructs in solvent systems suitable for phosphoramidite coupling (Rosenqvist *et al.*, 2024).

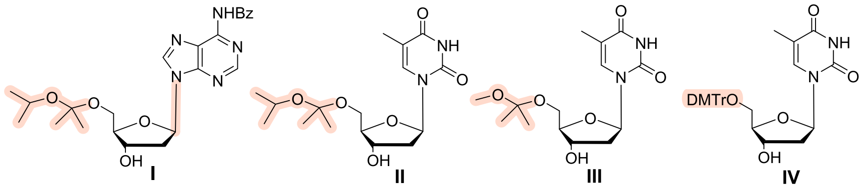
the protecting groups (39–67 m%) (Table 1A), which contributes markedly to the solubility of the soluble support-oligonucleotide construct, the solvent volume of the reactions and, consequently, to the reaction efficiency on the pentaerythritol core. The solubility of the constructs in organic solvent systems could be improved by varying the nucleobase protecting group scheme [isobutyryl (Gua), 1-butylpyrrolidin-2-ylidene (Gua, Cyt), 2,4-dimethylbenzoyl (Ade, Cyt), and N^3-Bz (Thy)]. It should be noted that as the oligonucleotide chain lengthens, the mass concentration of the soluble support construct must increase (cf. black bars in Fig. 15). This is a challenge for the use of larger constructs in solvents systems commonly used for LPOS. Ferguson *et al.* studied solubility properties of the PEG-oligonucleotide constructs in OSN-based LPOS [109]. During synthesis of an 18-mer phosphorothioate oligonucleotide, the solubility of the PEG (20 kDa)-support-oligonucleotide construct in acetonitrile was noticed to gradually decrease (from 210 to 18 g l^{-1}) due to the increased oligonucleotide content. By choosing the right size of the tetrapodal PEG support (PEGs of 5, 10, 20, and 40 kDa studied), it was possible to maintain the sufficient solubility, synthetic efficiency and filtration properties of the soluble support-oligonucleotide construct.

For the further development of LPOS, attention should be paid to the use of alternative protecting groups for nucleobases. As mentioned above, amidine protection of exocyclic amino groups and blocking of the N^3-H of thymine can increase lipophilicity [111, 112, 142]. Takeda acylated nucleobases using alkyl chain-modified gallic acid [143]. In addition to alternative protecting groups, *N*-unprotected oligonucleotides could find applications in convergent approaches (cf. Figs 5 and 10). In early reports, *O*-selective phosphite triester [144], phosphotriester [145], and phosphoramidite [146] coupling between *N*-unprotected nucleosides was used for the classical solution-phase synthesis of short oligonucleotides (<10-mers). Since then, the theoretical background and parameters for *O*-selectivity have been extensively studied, leading to development of efficient SPOS methods without the need for nucleobase protection [147–150]. Further improvements in convergent assembly (cf. Fig. 10) might lead to recognition of the advantages of the *N*-unprotected base approach, as different solubility/precipitation properties of protected and *N*-unprotected oligonucleotide fragments can be utilized for the purification.

Role of 5′-*O*-protecting groups in liquid-phase synthesis

DMTr is an ideal 5′-*O*-protecting group for SPOS. It can be orthogonally removed by mild and fast acid treatment with 1%–3% di- or trichloroacetic acid in dichloromethane or toluene [151]. The reaction follows the SN1-mechanism in which the protonated 5′-*O*-ether acts as a leaving group resulting in the formation of a stable DMTr cation. On a solid phase, continuous washing speeds up this reversible reaction (completed in seconds) and a simple colorimetric detection of the released DMTr cation can be used to minimize the time of acid exposure. The 5′-*O*-DMTr-protection has also been used in LPOS, but issues may be encountered due to the reversibility of the cleavage reaction. In solution, detritylation is dependent on the concentration, scale, and sequence of the oligonucleotide. Therefore, the acid treatment needed to complete the reaction in solution may lead to significant depurination, i.e. acid-catalyzed *N*-glycosidic bond cleavage of adenine and guanosine residues. The other side reactions are acid-catalyzed deamination [152] and premature N^4-acyl deprotection of cytosine residues [32, 129]. The products of acid-catalyzed deamination can remain hidden in standard analytics due to their structural similarity to the desired product. The products of premature N^4-acyl deprotection lead to the generation of phosphoramidate adducts in the subsequent coupling reaction [32]. Depurination can be observed as chain breakage or, more likely, as a replacement of the purine base by other aglycons, for example, by methanol in the

Table 2. Acid-catalyzed methanolysis of IIP, MIP, and DMTr-protecting groups and depurination (Liang *et al.*, 2023).



Nucleoside	5'-O-deprotection $T_{1/2}$ / s	depurination $T_{1/2}$ / s
I	6 ± 1	25000 ± 110
II	10 ± 2	NA
III	125 ± 6	NA
IV	560 ± 54	NA

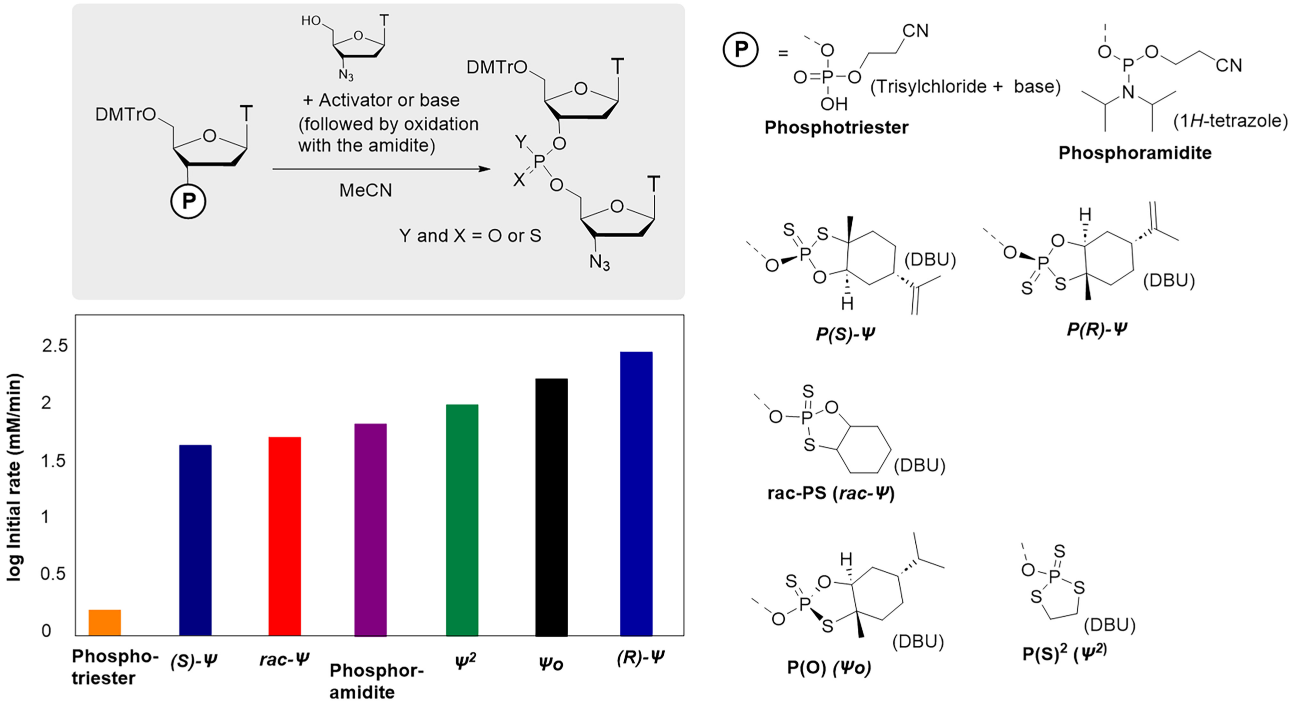
Reaction conditions: 6% formic acid in DCM-MeOH (2:1, *v/v*), r.t..

deprotection cocktail [153, 154]. Depurination is faster with 2′-deoxyribonucleotides than with ribonucleotides [155]. In fact, the synthesis of 2′-*O*-modified RNA can be done safely without depurination even under relatively harsh acid treatment conditions. DMTr cation scavengers such as pyrrole [31–33], thiols [32, 121], and silanes [108] are used to overcome the reversibility issue in solution, but scavengers as such do not change the reaction rate difference between detritylation and depurination. Walther and coworkers synthesized 20-mer homo 2′-deoxyadenine sequences on a tetrapodal PEG soluble support and investigated in detail the crucial parameters for detritylation (Fig. 6B) [108]. Interestingly, a 6100-fold rate difference between detritylation and depurination was achieved in a high acid and low temperature reaction. In a one pot coupling-detritylation, DCA and triethylsilane (70 and 25 equiv. to DMTr groups, respectively) were added to the coupling cocktail in acetonitrile at 0°C. After 110 min, the deprotected product was precipitated in 2-propanol. Using this acid treatment, consisting of 13% DCA in the total reaction volume, depurination was efficiently prevented. 5′-Terminal purine nucleosides are more susceptible to depurination than intrachain 2′-purine nucleosides [154, 155]. Walther incorporated a "thymidine cap" in oligo dA sequences, exposed these sequences to prolonged acid treatment, and concluded that internal dA remain mainly intact and depurination occurs primarily on terminal dA unit. While the issues with acid treatment appear to have been partially resolved, the other shortcoming of DMTr is its lipophilic derivatives, which form after deprotection. These can be difficult contaminants to deal with in LPOS-compatible procedures. As a result, alternative 5′-*O*-protecting groups [156], with better orthogonality and more traceless removal, could be valuable for LPOS. Two acetone acetals, MIP [63, 64, 113, 125, 134] and 5′-*O*-(2-isopropoxypropyl) (IIP) [157], have been suggested as alternative protecting groups. The pseudo irreversible and fast acid-catalyzed removal of MIP and IIP yields volatile byproducts: acetone, methanol, and isopropanol. Deprotection rates of MIP and IIP in acidic alcoholysis are ∼4.5- and 56-fold higher, respectively, than those of DMTr (Table 2). Considering the reaction rates and easily removable byproducts, MIP and IIP might be more attractive as 5′-*O*-protecting groups for LPOS. However, because of their higher acid sensitivity, these protecting groups are susceptible to premature cleavage under the slightly acidic conditions used for phosphoramidite coupling, which may result in uncontrolled elongation of the sequence (i.e. formation of $n + 1$ products). This is also observed with DMTr-protected building blocks when long coupling times are used [132]. With proper selection of activators and appropriate adjustments in their stoichiometry, premature deprotection of MIP and IIP can be prevented. Phosphoramidite coupling can also operate well in a mixture of pyridine and acetonitrile, in which acid catalysis is milder [125, 158]. No issues were observed when MIP was used in Ψ-building blocks [64], in which coupling is performed under basic conditions (Fig. 12). It may be worth noting that a regioselective acetalization method for the preparation of 5′-*O*-MIP-protected nucleoside building blocks has recently been reported [159], which makes MIP a more sustainable 5′-*O*-protecting group choice for LPOS.

So far, no other alternative 5′-*O*-protecting groups have been used in LPOS. The narrow reaction rate window between depurination and removal of acid labile protecting groups (DMTr, MIP, and IIP) is still a problem. Better orthogonality could facilitate synthesis of 2′-deoxyribonucleotides. The azidomethyl group has been successfully used as a 3′-*O*-protection in the controlled polymerase-catalyzed assembly of oligonucleotides [160] and as a 2′-*O*-protection in chemical RNA synthesis [86, 161]. Its fast removal by mild reducing agents such as tris(2-carboxyethyl)phosphine could make it attractive for 5′-*O*-protection too.

Importance of coupling chemistry in liquid-phase synthesis

In solution-phase synthesis, all conventional chemical chain elongation techniques, including phosphoramidite, phosphotriester, and *H*-phosphonate chemistries, can be used (Fig. 4). Phosphoramidite chemistry [21] is the most popular due to the commercial availability of the building blocks and its established fast coupling rates. However, the coupling rate does not correlate with reagent consumption. Phosphoramidites are prone to hydrolysis to inactive *H*-phosphonates (Fig. 4A). This is an issue if the goal is to perform couplings with a small excess of building blocks. The other shortcoming is the need for two-step coupling, i.e. formation of the phosphotriester intermediate and its subsequent oxidation, since it complicates the practical implementation of synthesis. In this respect, phosphotriester and *H*-phosphonate chemistries that can be performed in the presence of *in situ* activators is advantageous. *H*-phosphonates can be activated *in situ* by anhydrides



The activators or base shown in parentheses.

Figure 16. Relative coupling rates of Baran's P(**V**) building blocks (Huang *et al.*, 2021).

or acyl chlorides [162–164] (Fig. 4B) or oxidatively by electrophilic activators, such as *N*-bromosuccinimide, iodine, and carbon tetrachloride (Fig. 4C) [106, 165, 166], and phosphodiesters can be activated by arylsulfonyl chlorides or arylsulfonyl azolides (Fig. 4D) [101, 102, 167]. Hydrolysis in these coupling reactions results in reformation of the starting material. Therefore, the activity of the monomers can be maintained with an excess of activators even in the presence of a small amount of water. Furthermore, slightly basic conditions can be used, which prevents premature cleavage of the acid labile 5′-*O*-protecting groups (discussed above). The major advantage of phosphotriester and oxidative *H*-phosponate chemistries is that they require only one-step coupling. The omission of the oxidation step simplifies their practical implementation, prevents side reactions associated with oxidation, and can provide cleaner products after LPOS-compatible workups. Ikehara *et al.* applied a fundamentally different phosphotriester-based approach for the synthesis of oligonucleotides in solution [79–81], which could find interesting applications in modern LPOS methods. In this approach, an inert phosphoranisidate moiety is introduced into the nucleoside, where it acts both as a temporal protecting group and as a precursor of the phosphate. The anisidate group can be removed by organonitrites or nitrite salts, which expose the 2-chlorophenyl protected phosphodiester for phosphotriester coupling (Fig. 4F).

The reactivity of phosphorus correlates with its oxidation states [P(III) versus P(V)]; for example, trivalent phosphoramidites (P(III)) are generally more reactive than pentavalent phosphotriesters (P(V)). Although not of utmost importance in solution-phase synthesis, the coupling rate of phosphotriesters can be increased by an intramolecular nucleophilic catalyst. In Efimov's phosphotriester method, 4-methoxy-1-*N*-oxido-2-picolyl is used as a phosphate protecting group [168]. The intramolecular nucleophilic *N*-oxide catalyst of the group results in a marked increase in the reaction rate that is comparable to that observed with phosphoramidites. Baran and coworkers reported recently a fundamentally different P(V) approach (cf. the toolbox in Fig. 16). In addition to reactive species for stereo-controlled synthesis of phosphorothioates (*P(R)-Ψ* and *P(S)-Ψ*), building blocks for racemic phosphorothioates (*rac-Ψ*), phosphorodithioates (*P(S)*^{2}), and phosphodiesters (*Ψo*) have also been developed. It is notable that the reaction rates of these P(V) building blocks are similar or even slightly higher than those of phosphoramidites.

The phosphate protecting group merits attention in LPOS. Although not tightly associated with a specific coupling chemistry, cyanoethyl and 2-chlorophenyl phosphate protecting groups are usually used with phosphoramidite chemistry and phosphotriester chemistry, respectively. Cyanoethyl groups are prone to premature cleavage during LPOS [32, 129, 132]. Although this is not a critical side reaction affecting chain elongation efficiency [32], it increases the hydrophilicity of the growing oligonucleotides, which interferes with their isolation in LPOS-suitable work-ups (extraction and precipitation). Such a problem has not been observed with, for example, the lipophilic and relatively stable 2-chlorophenyl phosphate protecting group (removed by aldoximes at the end of synthesis) [105, 135]. Baran's P(V) chemistry results in an exposed phosphate or phosphorothioate backbone, which impedes the isolation of the growing oligonucleotide products in LPOS.

Advances in liquid-phase synthesis of PMO and PNA

The chemical structure and solubility properties of PMO and PNA are substantially different from those of natural nucleic

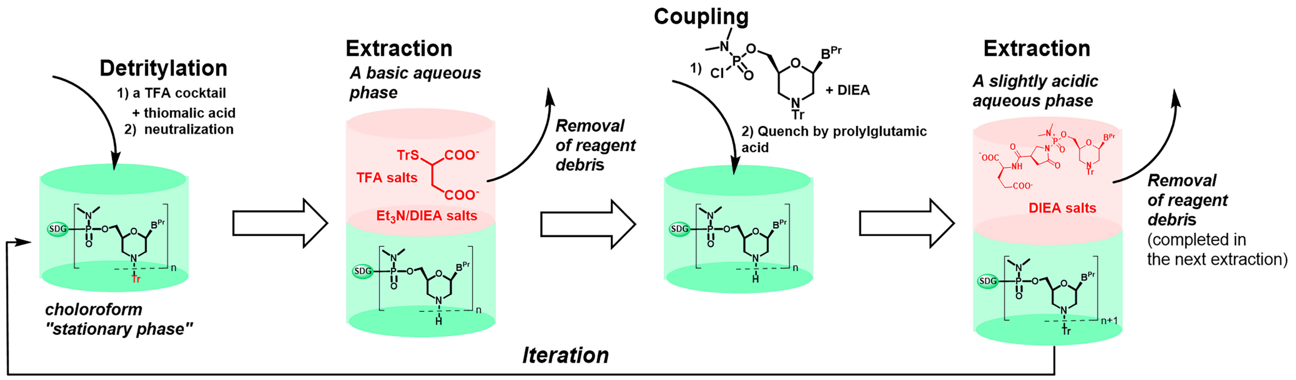


Figure 17. Principle of the continuous extraction system used for liquid-phase synthesis of PMOs.

acids, as is the chemistry (i.e. phosphoramidate and amide couplings) used for their chain elongation. Liquid-phase synthesis of PMO and PNA is therefore discussed separately in this section. PMO synthesis by Ajiphase (Fig. 9C) follows a continuous liquid-liquid extraction procedure (Fig. 17) like the one used for peptide synthesis. A galloyl-derived lipophilic SDG is applied, and chain elongation proceeds in the 5′→3′-direction (i.e. reversed order in comparison to LPOS). In this procedure, the secondary amine of the morpholino ring attacks the 5′-O-chlorophosphoramidate moiety of the next PMO building block. The synthesis cycle occurs in a chloroform stationary phase and is initiated by detritylation using a trifluoroacetic acid (TFA) cocktail. Thiomalic acid is used as the trityl scavenger, resulting in the formation of a hydrophilic trityl derivative. After detritylation, the chloroform mixture is neutralized and washed with mixtures of aqueous Na_2CO_3-DMF and brine-DMF. The organic phase is separated and a slight excess of chlorophosphoramidate PMO building blocks in the presence of diisopropyl ethylamine (DIEA) is added. The coupling is quenched by the addition of prolylglutamic acid (Fig. 17), and washing is performed with a mixture trifluoroethanol and aqueous HCl. The separated organic phase is exposed to the next detritylation. An example of the synthesis of a 9-mer PMO is shown in Fig. 9C. The fully protected and SDG-bound PMO product, obtained with a crude yield of 72%, is exposed to global deprotection using concentrated ammonia.

Because of the amide backbone, PNAs can be synthesized by the soluble support-assisted liquid-phase methods developed for peptides [110], including thermomorphic liquid–liquid extractions [169], modern nanofiltration [170, 171], and magnetic separation [172, 173]. Activated PNA monomers have, however, one characteristic that distinguishes them from their α-amino acid counterparts, i.e. they can readily form ketopiperazines (cf. structure in Fig. 9D and diketopiperazine formation of dipeptides) [174], which facilitates one pot coupling-deprotection cycles during liquid-phase synthesis. Chiba *et al.* introduced a liquid-phase synthesis method for the preparation of PNAs (Fig. 9D). The carboxy terminus of PNAs was protected by a hydrophobic benzyl alcohol SDG [175]. A mixture of Fmoc/Bhoc (fluorenylmethoxycarbonyl/benzhydryloxycarbonyl)-protected PNA monomers, COMU (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate), and DIEA in THF was used for each coupling. After an appropriate coupling time, a mixture of DBU and piperidine was added directly to the coupling cocktail to remove Fmoc and to result in simultaneous ketopiperazine formation of the activated PNA monomers used in excess (1.1–1.5 equiv.). One precipitation per synthesis cycle was used without uncontrolled elongation of the sequence. A protected heptamer PNA was obtained with an overall yield of 79% over six consecutive couplings, deprotections and precipitations in acetonitrile, and then globally deprotected using a TFA cocktail. It is worth noting that protected PNAs are particularly susceptible to aggregation, and further development of liquid-phase synthesis of PNA could benefit from an alternative protecting group scheme. As observed with oligonucleotides, blocking of N^3-H of thymine can abrogate hydrogen bonding and aggregation [176]. In some cases, good synthetic efficiency has been achieved without exocyclic amino protection of purine bases, which reduces the amphipathic nature and aggregation of PNA [177].

Liquid-phase synthesis of fragments suitable for enzymatic or chemical ligation

Considering the potential solubility/reactivity issues of soluble support constructs, as well as the accumulation of side products ($n-1$, $n+1$ products, derivatives of depurination, and protecting group specific ones), it seems reasonable to suggest that the LPOS methods are competitive, in comparison to SPOS and modern biocatalytic methods, for the large-scale synthesis of relatively short chemically modified fragments. In most reports, short (shorter than 10-mers) oligonucleotides have been synthesized. However, the short oligonucleotide fragments obtained by LPOS can be used as starting materials for different hybridization-driven ligations to yield long sequences. This approach can achieve efficient synthesis and efficient downstream processing without the need for chromatography. Furthermore, quality control can be improved, since analyses can focus on the product produced from well-characterized blocks rather than on the product contaminated by side-products of single unit or internucleotidic differences. The latter may be particularly critical for the synthesis of stereo-controlled phosphorothioate oligonucleotides. The next question is, should ligation be enzymatic or chemical? Marked progress has been made in template-dependent ligase and chemical phosphodiester ligation methods, discussed in the following sections. The potential of the template-dependent ligase method has been verified on an in-

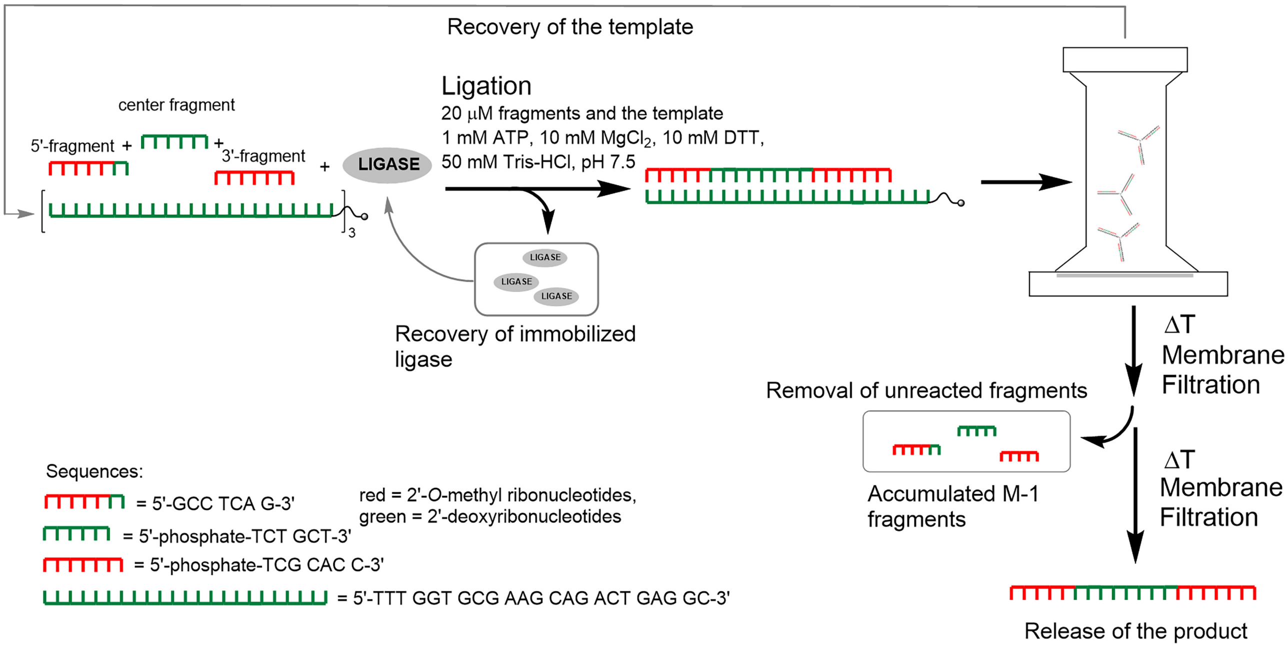


Figure 18. Assembly of a gapmer oligonucleotide by an engineered DNA-duplex ligase (Crameri and Tew, 2019). The ligase is immobilized on to a cellulose bead and is separated from the reaction mixture and reused. Membrane filtration is utilized to recover the fragments and the tripodal template oligonucleotide.

dustrial scale [56–58], but chemical phosphodiester ligation has been restricted to diagnostic applications and academic interest so far. Terminal phosphate groups must be incorporated into fragments before the fragments can be ligated. A 5′-monophosphate (or thiophosphate) is required for the linking of fragments by ligases [178–188], whereas 3′-terminal phosphate groups are preferred for chemical ligation. For the incorporation of 5′- and 3′-phosphates into fragments, appropriate reagents and linker structures are available, which can be used as an integral part of the chemical chain elongation.

Ligase-assisted assembly of oligonucleotides: a benchmark for chemical ligation

The utility of using ligases to produce oligonucleotides from shorter fragments was already demonstrated in the late 1960s [189], and this has been extended to produce functional genes [190]. The efficiency and greenness of using ligases has attracted much attention in recent years as a sustainable alternative for the manufacture of chemically modified oligonucleotides [1, 2, 35, 36], including medium- to large-sized (several kilobases long) oligonucleotides [47–55, 191, 192]. The examples presented in this section demonstrate the power of using ligases for the manufacture of chemically modified oligonucleotides, and for the facile production of nucleic acid libraries. The efficiency of coupling by ligases can be considered a benchmark for judging the efficiency of chemical ligation methods. Furthermore, technical innovations in the purification and isolation of the products and the production of libraries using ligases could also be adopted in advanced chemical ligation methods, which are discussed in the following sections.

Glaxo Smith Kline described a technology platform for the template-driven ligation of crude chemically synthesized oligonucleotide fragments. Engineered double-stranded DNA ligases immobilized on cellulose beads were utilized [56]. An example of gapmer oligonucleotide synthesis based on this platform is described in Fig. 18. The assembly consists of three fragments: a 5′-fragment (7-mer), a 5′-monophosphorylated center fragment (6-mer), and a 5′-monophosphorylated 3′-fragment (6-mer) together with a tripodal complementary template oligonucleotide on a PEG-homostar soluble support (cf. **II**/Fig. 11). Simultaneous template-driven ligation of multiple fragments is performed in the presence of adenosine triphosphate (ATP) in Tris–HCl-buffer (tris(hydroxymethyl)aminomethane, pH 7.5. After conjugation, the ligase is removed by centrifugation or filtration and reused. Gradual annealing and membrane filtration are used to separate the tripodal template, unreacted fragments, and the product. The filtered tripodal template can be reused. Crude oligonucleotide fragments containing single unit insertions or deletions can be used in this technology, due to the different T_m-values between the guiding template and the fragments of variable length. The final product is obtained at higher purity than that of the fragments and is sufficient for therapeutic applications. Chromatography-based downstream processing is not required. In an alternative method, biotin-conjugated template oligonucleotides, which can be immobilized on streptavidin-coated particles, was used following similar separation techniques, including gradual annealing of the unreacted fragments and the product. It is important to realize that Watson–Crick base pairing has dual roles in this platform; on the one hand, it controls ligase-mediated assembly and on the other, it assists in the purification of the product. In summary, it is possible to achieve efficient and scalable self-purification-based preparation of therapeutically relevant oligonucleotides using the above protocols. Many different oligonucleotides, including those with modification of the phosphodiester and phosphorothioate backbone and sugar moieties, can be prepared using this platform.

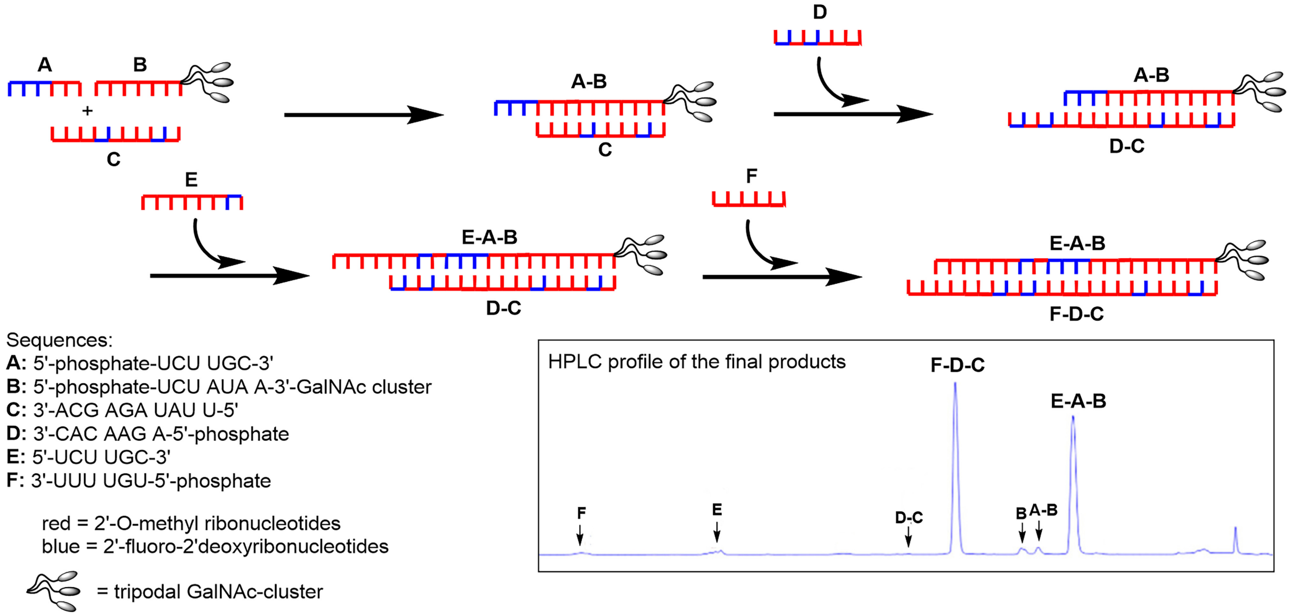


Figure 19. Assembly of an siRNA using an engineered RNA-duplex ligase in a one pot system. Adapted with permission from {Paul, S., Gray, D., Caswell, J., Brooks, J., Ye, W., Moody, T. S., Radinov, R., Nechev, L. Convergent Biocatalytic Mediated Synthesis of siRNA. *ACS Chem Biol*, **2023**, *18*, 2183–2187}. Copyright {2025} American Chemical Society.

Almac and Alnylam applied a convergent biocatalytic synthesis strategy using a hybrid RNA ligase for the preparation siRNAs (Fig. 19) [57, 58]. This strategy depends on overlapping ligation sites, in which the assembled antisense and sense strands act as templates for each other. Ligation sites and different ligases were screened to find optimal productivity and purity of the target siRNA. Six fragments, one containing a trivalent *N*-acetyl galactose (GalNAc) cluster, were successfully ligated in a stepwise one pot system. The applicability of crude chemically synthesized fragments for the assembly was evaluated. Almac group also used RNA ligases for template-independent ligation of oligonucleotide fragments (https://www.almacgroup.com/knowledge/wp-content/uploads/sites/10/2021/04/Man-Chem-Enzyme-synthesis-16.03.21-1.pdf).

In this case, the 3′-terminal phosphate was used as a temporal protecting group to prevent uncontrolled polymerization. After ligation, the 3′-*O*-phosphate cap was removed by phosphatase [37]. Template-dependent ligation was superior to template-independent ligation in that it allowed self-purification of the products.

Recently, Genentech-Roche presented an efficient ligase-assisted approach for the production of single guide RNAs, essential reagents for CRISPR–Cas9-based gene therapy applications [193]. In optimized conditions, near stoichiometric amounts of a 49-mer 5′-fragment (1.0 equiv.) and a 51-mer 5′-monophosphorylated 3′-fragment (1.1 equiv.), synthesized by SPOS, were ligated on a 28-mer DNA template (1.2 equiv.) using T4 RNA ligase 2 in the presence of ATP and $MgCl_2$ in Tris–HCl buffer (pH 7.2). The process produced a variety of single guide RNAs on a decagram scale for CRISPR–Cas 9 applications. Higher purity and 3–4 times greater yields of the products (100-mer RNA) were achieved compared to traditional linear SPOS.

Herdevijn *et al.* evaluated the applicability of T4 DNA ligase for the ligation of modified oligonucleotides under crowded reaction conditions, including fragments consisting of 2′-OMe ribonucleotides, locked nucleic acids, FANAs (2′-fluoro arabinonucleic acids), and hexitol nucleic acids [51]. Template-driven ligation between the fragments (0.1 μM acceptor, 0.2 μM donor and template, and one ligation site) led to high conversion of the products under optimized conditions, and the crowded conditions (achieved by adding DMSO, betaine, or PEG8000) were shown to increase the efficiency of ligation.

Hollenstein *et al.* reported the efficient assembly of chemically modified oligonucleotides using pentanucleotide 5′-monophosphate fragments as substrates for T3 DNA ligase [54]. Scaled access to a broad range of modified oligonucleotides of up to 120 nucleotides, including clinically relevant 20-mer antisense oligonucleotides (analogs of Fomivirsen), was successfully synthesized. A biotin-conjugated DNA template was used to guide the assembly and facilitate isolation of the products by magnetic separation on streptavidin-coated beads. The assembly of a Fomivirsen LNA gapmer analog was achieved using this technology (described in Fig. 20). One pot ligation at 4°C for 24 h with a slight excess (1.4 equiv.) of four fragments, three pentamers and one hexamer, and a template resulted in a product with a yield and purity of 71% and 85%, respectively.

Ligase-catalyzed oligonucleotide polymerization (LOOPER) introduced by Hili *et al.* [194] is not primarily designed for synthetic purposes, but it highlights the versatility and efficiency of ligases to produce modified oligonucleotide libraries. For example, it has been used to increase the chemical diversity and functionality of aptamers [47]. To produce an aptamer library, chemically modified 5′-monophosphate fragments (pentamers) were assembled by T4 DNA ligase on a biotin-modified DNA template consisting of an eight-codon encoding region (8 × 5 nt = 40 nt long) between an initiator and a terminator primer (Fig. 21). Each fragment had a modified dA unit with variable functionalities at C8. The library was released from the template by denat-

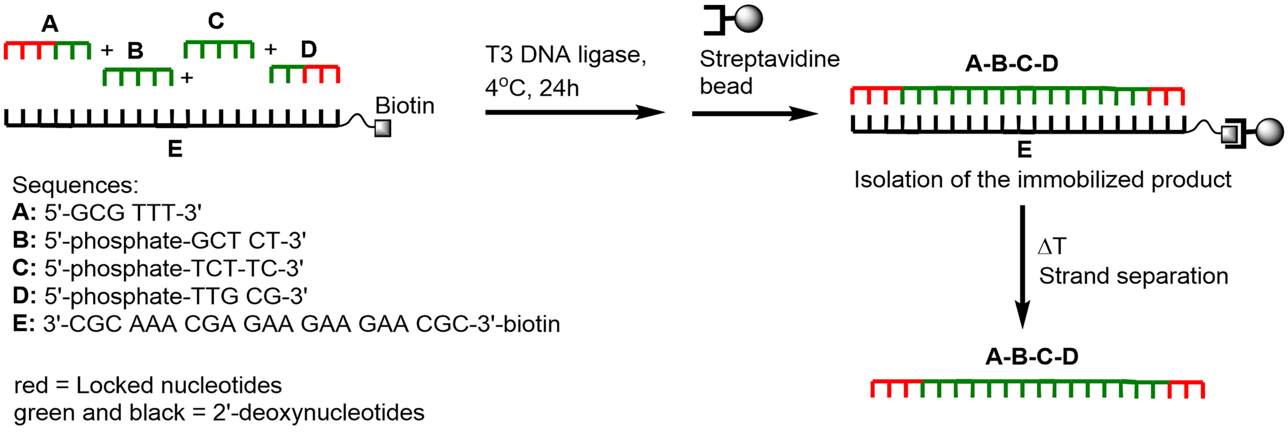


Figure 20. T3 DNA ligase-dependent one pot assembly of a Fomivirsen analog using four fragments.

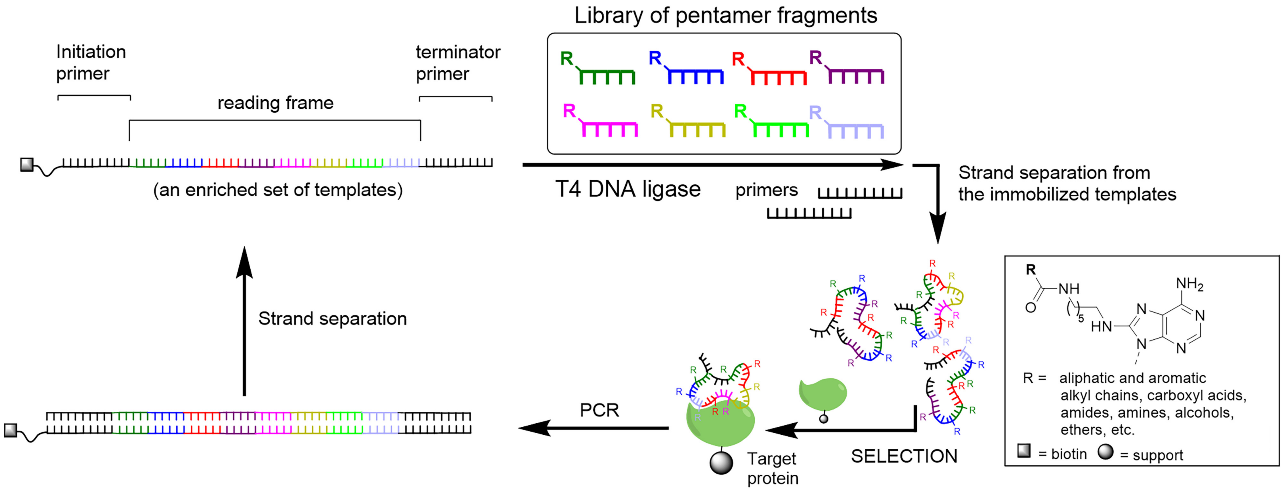


Figure 21. The idea of using LOOPER to create libraries of chemically modified aptamers (Hili *et al.*, 2013).

uration and exposed to positive selection pressure with the target protein. The bound aptamer candidates were released from the substrate by denaturation, amplified by PCR using KOD polymerase (a thermostable DNA polymerase from *Thermococcus kodakaraensis*) in the presence of a biotin-modified primer, leading to a library of selected templates. Iterative rounds of LOOPER/SELEX (Systematic Evolution of Ligands by Exponential Enrichment) were performed to decrease the number of potential candidates in the modified aptamer pool. In this way, a high-affinity chemically functionalized human α-thrombin aptamer was produced.

Use of templated chemical ligation for phosphodiester bonds

The phosphodiester bond can form chemically between unprotected oligonucleotide segments in aqueous media in the presence of a template oligonucleotide. As with ligases, the assisting template (i.e. splint) oligonucleotide accelerates the reaction rate, prevents hydrolysis of the activated phosphate, and controls the regioselectivity of phosphodiester formation through a hybridization-driven proximity effect [195, 196]. DNA-templated phosphodiester ligation was described for the first time almost 60 years ago by Naylor and Gilham [197]. Carbodiimide activation was used for Poly-dA-templated ligation between 3′-OH and 5′-phosphate groups of thymine penta- and hexanucleotides with modest yields. Since then, potential condensing agents, including carbodiimides [198–202], carbonyl diimidazole [203], cyanogen bromide [69, 204, 205], and its derivatives cyanimidazole and cyanamide [206], as well as nucleophilic catalysts/additives [207–209] and the role of metal ions in the reaction have been studied, leading to improved ligation efficiency. Template-driven phosphodiester bond formation on higher order secondary structures has also been examined [210, 211]. Hud *et al.* re-evaluated the critical parameters of carbodiimide-induced ligation of penta- and nonamer DNA substrates (50 or 1.5 equiv.) on a DNA-hairpin template (1.3 µM) using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 250 mM) and $MnCl_2$ (5 mM) in a MES (2-(*N*-morpholino)ethanesulfonic acid, 100 mM, pH 6) buffer (Fig. 22) [212]. The favored reaction direction was opposite to that of the ligases due to 5′-OH→3′-phosphate-ligation being faster than the 3′-OH→5′-phosphate-ligation. The 5′-OH→3′-phosphate-ligation is faster because the nucleophilicity of the primary 5′-OH group is higher than that of the secondary 3′-OH group. Longer substrates (5- versus 9-mer) with stronger hybridization increased the reaction rate and

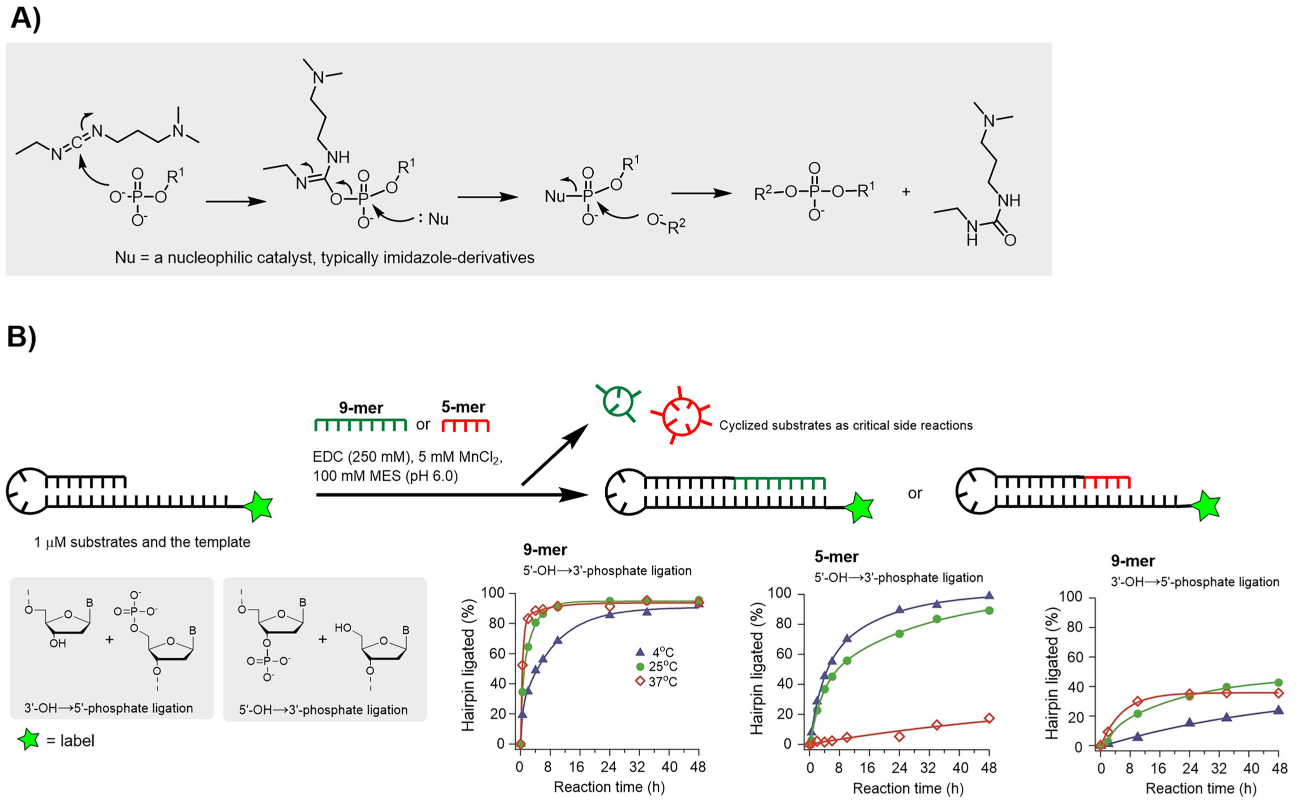


Figure 22. Mechanism of EDC-induced phosphodiester formation (**A**). EDC-induced ligation of 5- and 9-mer DNA fragments on a hairpin template (**B**). Adapted with permission from {Obianyor, C., Newman, G., Clifton, B. E., Grover, M. A., Hud, N. V. (2020) Towards Efficient Nonenzymatic DNA Ligations: Comparing Key Parameters for Maximizing Ligation Rates and Yields with Carbodiimide Activation. *ChemBioChem*, **21**, 3359–3370} Copyright {2025} John Wiley and Sons.

reduced side reactions during ligation. The critical side reactions were head-to-tail cyclization and nucleobase modifications of the substrates (reaction of EDC to Gua and Thy) [213]. Both were less obvious when the substrate was bound to the template. Thus, increases in temperature (4°C, 25°C, and 37°C were examined), which increase the rate of the ligation, should be below the T_m-value of the substrate-template duplex. No marked role of divalent metal ions was observed. *N*-Ethylimidazole as a nucleophilic catalyst had no effect or reduced ligation efficiency. This was in contrast to EDC-induced template-driven chemical polymerization of nucleoside 5′-monophosphates, in which metal ions have an important catalytic role, as shown by Richert *et al.* [207, 208, 214, 215]. Kanaya and Yanagava introduced cyanogen bromide (BrCN)-induced activation, which in the presence of imidazole leads to ligation of oligoadenylates on a poly(U) template [204]. In the presence of imidazole, cyanogen bromide is converted to cyanimidazole or diimidazole imine [216]. Tertiary amines of buffers can also cause leaving group replacement [217–219]. On DNA models (0.2 mM), a mixture of cyanomidazole (250 mM), triethylammonium MES (10 mM, pH 6), and $MnCl_2$ (5 mM) resulted in efficient ligation [220]. On ribonucleotide models, the templated reaction controls not only the ligation efficiency but also regioselectivity between the 2′- and 3′-OH groups. The 2′-OH group of RNA is 6–9 times more nucleophilic than the 3′-OH group toward activated phosphate [221]. In template-directed ligation, in turn, the 3′–5′-linked phosphodiester is more favored than the 2′–5′-linked phosphodiester for the organized stereochemical orientation of the fragments [222, 223]. Divalent metal ions can affect 2′–5′/3′–5′-selectivity [204] and accelerate condensation. Okita *et al.* studied the mechanism (Fig. 23A) and optimized the conditions of cyanomidazole-mediated condensation (Fig. 23B) [70, 71]. Divalent metal ions (Cd^{2+}, Ni^{2+}, and Co^{2+}) increased ligation rates and the yields of DNA and threoninol nucleic acids (TNAs). The metal cation was suggested to facilitate formation of the activated phosphate and nucleophilic attack of the OH group to this critical and readily hydrolyzed intermediate (Fig. 23A). The ligation of DNA was fast but slower than that of TNA (Fig. 23B), and on the DNA, 3′-OH→5′-phosphate ligation was slower than 5′-OH→3′-phoshate ligation. Under optimized conditions (0.1 M NaCl, 5 mM $CdCl_2$, 20 mM cyanomidazole, and 1 μM oligonucleotide), an octamer primer was elongated by TNA trimers on a DNA template to produce a 29-mer TNA with a yield of 60% after 2 h of incubation at 4°C. This was a marked achievement as short trinucleotides could be efficiently polymerized in a controlled manner.

The examples above demonstrate that chemical phosphodiester ligation could be as effective as ligase-mediated ligation for the preparation of medium-sized oligonucleotides. If the assembly is based on only one or two ligation sites, and if the oligonucleotide product is relatively short, then no marked difference in yields is observed between chemical phosphodiester ligation and ligase ligation. Furthermore, relatively fast reaction rates with cheap condensation agents (in comparison to ligases and ATP, and considering their excess used) can be obtained, and no obvious limits for scalability can be seen.

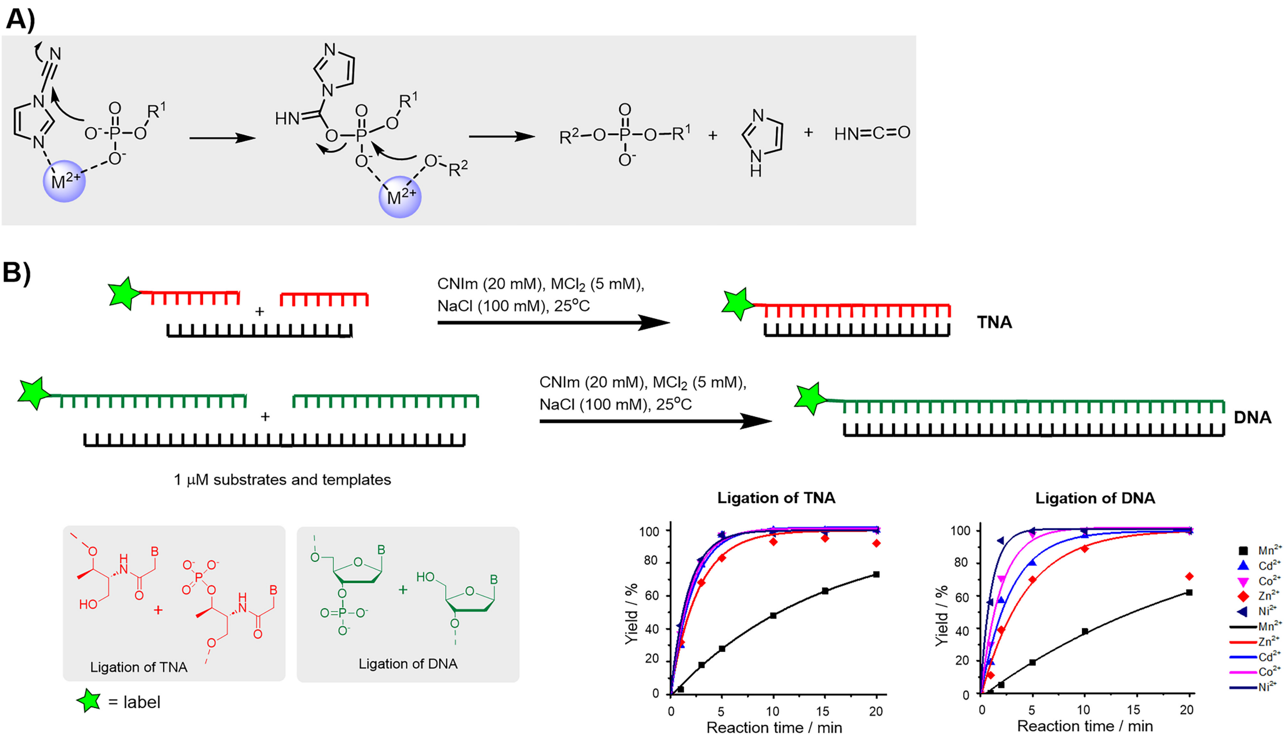


Figure 23. Cyanomidazole-activation of the phosphate and the role of divalent metal cations (**A**). Templated ligation of TNA and DNA fragments using cyanomidazole in the presence of different divalent metal ions (**B**) (Okita *et al.*, 2023).

There remains room for improving the efficiency of chemical ligation. Considerable research effort has been expended in developing agents that cleave phosphodiester bonds (i.e. artificial nucleases), but relatively little effort has been devoted to examining the potential of ligation catalysts (i.e. artificial ligases). An important question is, for example, could known cleaving agents [224] be used to accelerate the reverse reaction (i.e. ligation), if appropriate reaction conditions could be found? Transition state stabilization and potential catalysts that bring the nucleophile and the electrophile into close proximity [225, 226] could probably be utilized to accelerate ligation (Fig. 24).

Chemical ligation of artificial internucleotic linkages

Chemical and biological relevance of phosphoramidate, amide, amine, and triazoyl bridges

Because of competition between water and the hydroxy group of the nucleoside, phosphodiester bond formation is inherently difficult in aqueous reactions. In fact, there are a set of reactions (e.g. phosphoramidate formation, reductive amination, amide bond formation, and click reaction, cf. Fig. 25) that operate better than phosphodiester bond formation on a DNA template. The ease with which artificial bridges are formed in DNA templates has inspired research into areas as diverse as sequence-defined synthetic polymers, origin of life [196, 227–229], and DNA-encoded molecular libraries [6]. The demand for such backbone modifications will likely grow with the increasing interest in genome editing [230], synthetic biology [231], and "conventional" oligonucleotide therapeutics, including splice-switching oligonucleotides (SSOs), RNase *H*-active ASOs, and siRNAs.

The mechanisms of action of oligonucleotide therapeutics dictate specific limits on which modifications can be used. SSOs operate well with different backbone modifications. They act not by binding to critical binding pockets in enzymes, but by sterically blocking splicing factor binding to the pre-mRNA, leading to modulation of the splicing process. Therefore, SSOs with even heavily modified backbone structures can be used if the modifications do not disrupt Watson–Crick base pairing [232]. This has been thoroughly demonstrated with PMOs and PNAs. The cellular uptake and activity of the SSOs is known to be increased, when amide linkages are introduced into the middle of the phosphorothioate sequence [72]. RNase *H* is sensitive to chemical modifications in DNA and RNA and prefers a native phosphodiester or phosphorothioate backbone in DNA–mRNA–hybrid duplex substrates. Even minor modifications in the substrate, e.g. phosphoramidate linkages, can inhibit RNase *H* activity [233]. Brown *et al.* incorporated amide linkages into an ASO and evaluated RNase *H* cleavage of the target RNA [73]. No cleavage of the target RNA was observed when the amide linkage was placed close to the RNA binding pocket of RNase *H* (i.e. the 2′-deoxy window of the ASO). By contrast, cleavage of the RNA was observed when gapmer ASOs with amide linkages in their wing regions were employed. In a similar manner, phosphoramidate [234], PNA- and triazole-modified [235, 236] gapmer ASOs were only active when the modifications were placed in their wing regions. Interestingly, amide linkages are well tolerated at specific positions in siRNAs (Fig. 25C). Rozners *et al.* studied comprehensively the effects of systematic replacement of a single phosphate with an amide linkage throughout the guide strand of an siRNA [74, 75]. Amide linkages were well tolerated in the seed

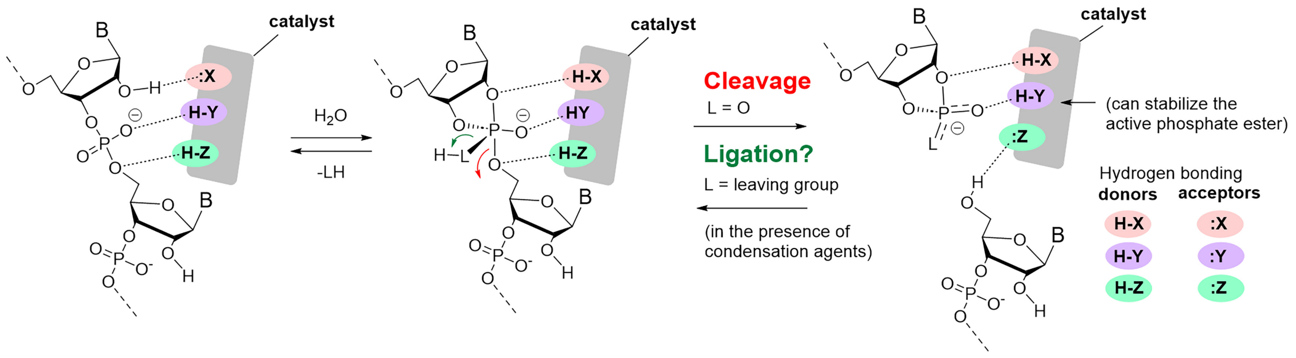
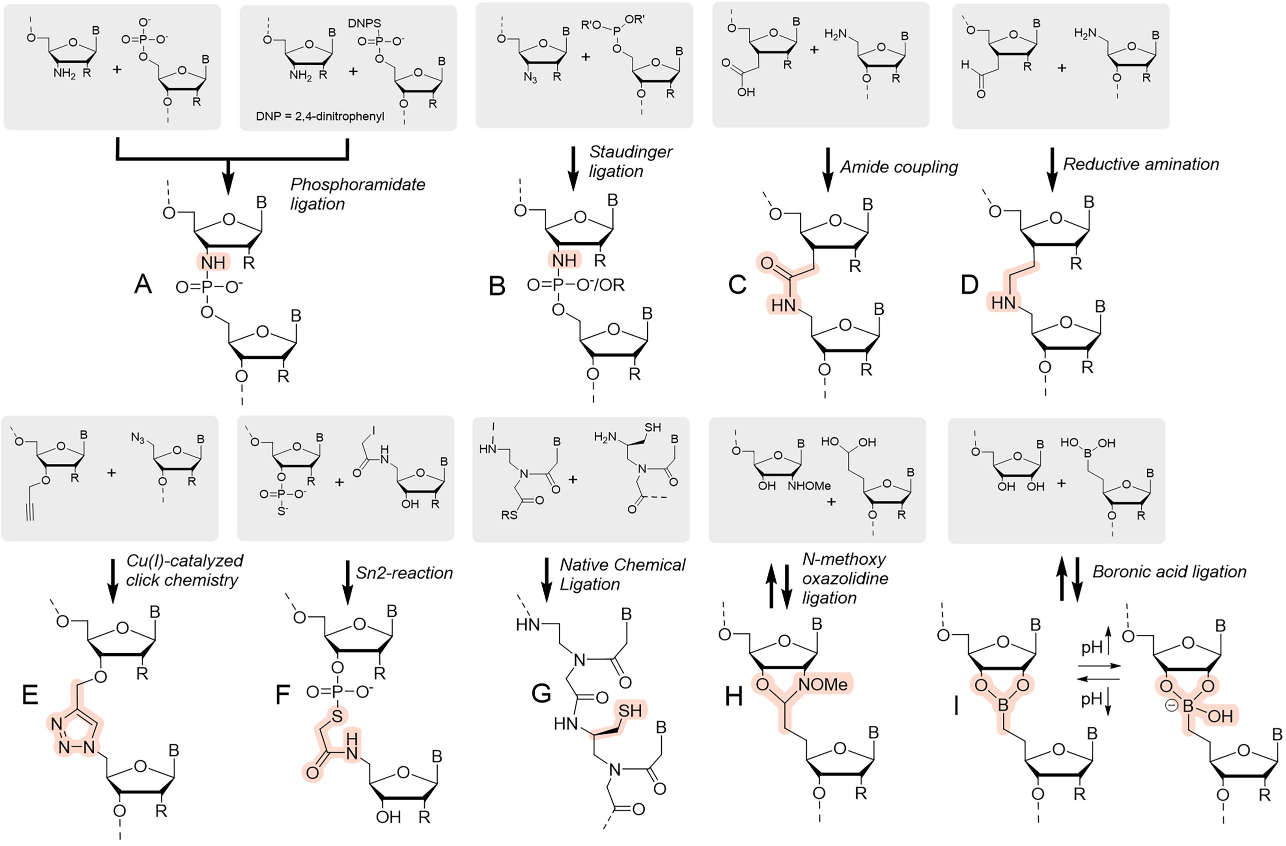


Figure 24. The key interactions of catalyzed RNA-hydrolysis could be utilized for ligation.



R = H or OH or 2'-O-modificaton

Figure 25. Examples of backbone modifications obtained by orthogonal ligations under aqueous conditions.

and central regions of the guide strand and even increased silencing activity when placed at certain specific positions in the catalytic site of Argonaute 2. A crystal structure and molecular dynamics simulations suggested that amides engage in hydrogen bonding with the protein and that this compensates for the absence of the critical phosphate-related interactions. Recently, the same group replaced the phosphate on the guide strand by a secondary amine (Fig. 25D). The modification destabilized the RNA duplexes thermally but was reasonably well tolerated in the guide strand of an siRNA with a relatively modest decrease in RNA interference (RNAi) activity [237].

These findings of the biological impact of artificial internucleotidic linkages in oligonucleotide therapeutics may encourage further research into the development new chemically modified oligonucleotide therapeutics, which in addition to conferring potential improvements in therapeutic activity, could also increase the robustness of templated chemical ligation of oligonucleotide fragments. Artificial internucleotidic linkages seem readily adaptable for use in SSOs, to a limited extent in RNase *H*-active ASOs, and in siRNAs. Considering the synthetic practicality, efficiency, and orthogonality of the reactions, as well as the biocompatibility of the artificial linkages formed, the focus should probably be on performing templated ligations yielding phosphoroamidate- [238–242], amide- [243, 244], secondary amine- [245–247], and triazole-derived [248–254] backbone modifications. The functional groups, azides, alkynes, amines, aldehydes, and carboxylic acids required for these backbone modifications can

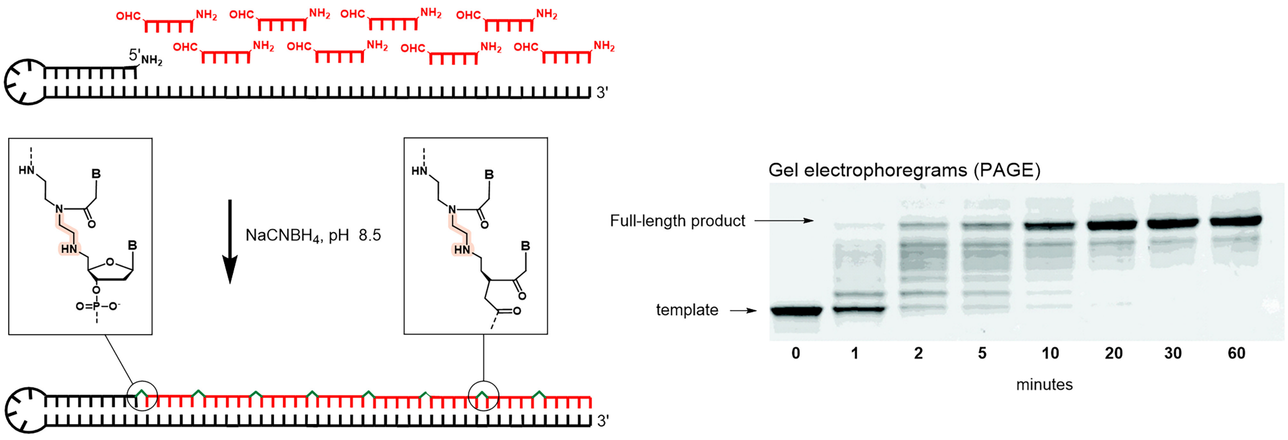


Figure 26. DNA-templated reductive amination for controlled polymerization of 5-mer PNA fragments. Adapted with permission from {Rosenbaum, D. M., Liu, D. R. (2003) Efficient and Sequence-Specific DNA-Templated Polymerization of Peptide Nucleic Acid Aldehydes. *J Am Chem Soc*, **125**, 13924–13 925}. Copyright {2025} American Chemical Society.

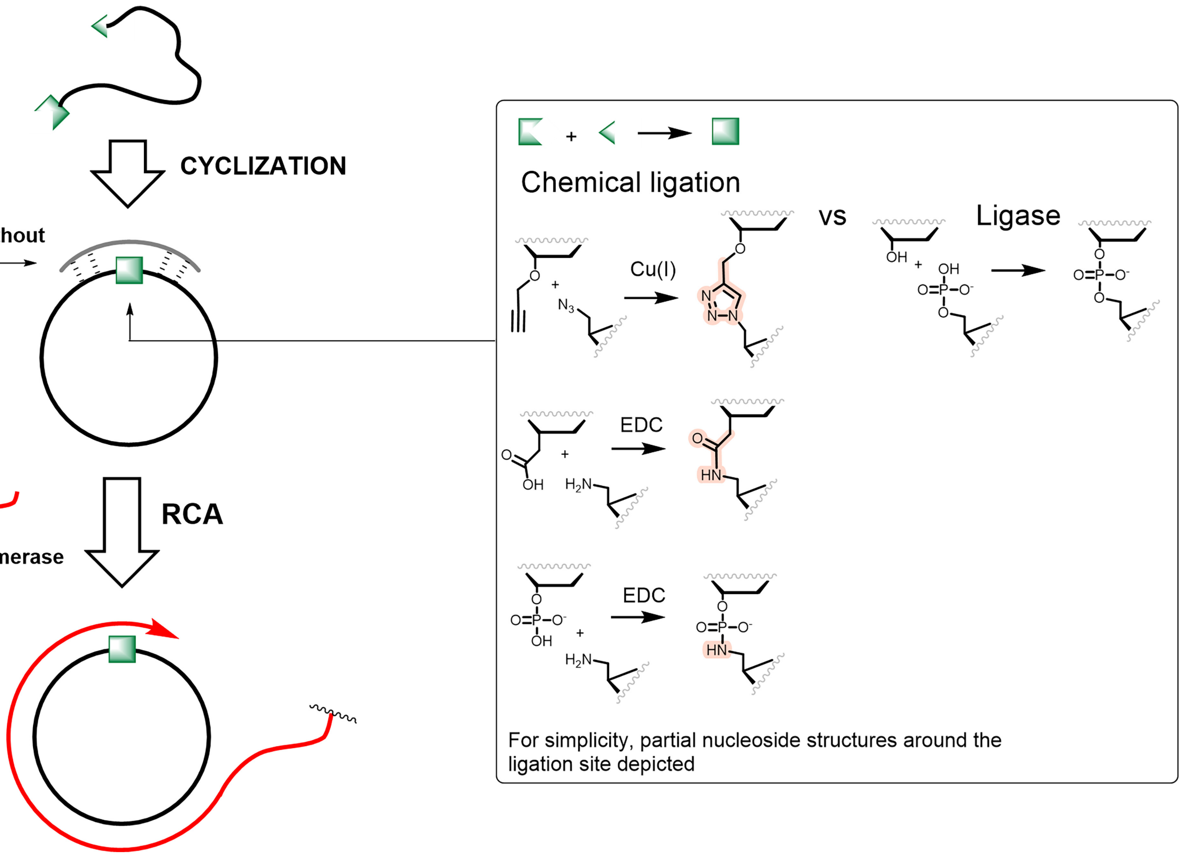
be readily introduced into oligonucleotide fragments. This is typically done by coupling an appropriately modified nucleoside monomer in the last step of chain elongation, and/or by using alternative linker structures, to yield the required 5′- or 3′-modification, respectively, in oligonucleotide fragments.

Reductive amination, yielding secondary amines, is a good example of an established orthogonal reaction that operates well on DNA templates. It has successfully been used for DNA-templated ligation of amino- and aldehyde-modified oligonucleotide fragments [245, 246, 255] and controlled templated polymerization of aminoaldehyde nucleoside analogs [256]. Liu and coworkers polymerized pentamer PNA aldehydes on a 5′-amino-modifed hairpin DNA-primer/template (Fig. 26) [257, 258]. Interestingly, the secondary amines formed in the reduction step resulted in reduced affinity for the template compared with imine intermediates [246]. As a result, no product inhibition was observed, indicating turnover of the template catalyst [259]. A 62-mer template (20 pmol) was annealed to 5-mer PNA aldehydes (each 4 equiv/match) in 0.1 M NaCl, 10 mM TAPS (*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid, pH 8.5) buffer and after exposure to $NaBH_3CN$ (80 mM) at room temperature, polymerization was followed by polyacryl amide gel electrophoresis (PAGE). Demonstrating the power of this ligation, near quantitative sequence-specific polymerization was observed in <1 h.

Orgel and coworkers evaluated the applicability of phosphoramidate, amide, and ester bond formation for DNA-templated ligation of oligonucleotide–PNA–chimeras [243]. Each of the ligations was performed using a 14-mer DNA or PNA template (0, 0.1, 1, or 10 equiv.), an 8-mer PNA substrate (0, 0.1, 1, or 10 equiv.) and a [^{31}P]-labeled 6-mer DNA-substrate (60 nM) in imidazole buffer (0.2 M, pH 7.7), and using EDC (0.11 M) as a condensation agent. Different directions of ligation were evaluated: 3′-phosphoramidite ligation (i.e. reaction between the 3′-monophosphate group of a DNA fragment and the terminal amine of a PNA fragment) was efficient, whereas 5′-phosphoramidate ligation was inefficient and led to a modest yield. The 3′-phosphoramidate ligation on a DNA template also resulted in catalytic turnover: 40% of the chimeric product was observed after 7 days at 25°C when 0.1 equiv. of the DNA template and 1 equiv. of the PNA substrate were used for ligation. No turnover was observed with the PNA template. The initial rate of amide bond formation (i.e. reaction between a 3′-amino DNA and a carboxyethyl-modified PNA) was almost as high as that of 3′-phosphoramidate ligation, but produced side products, especially during long reaction times. Ester bond formation was inefficient and resulted in detectable products only at 0°C.

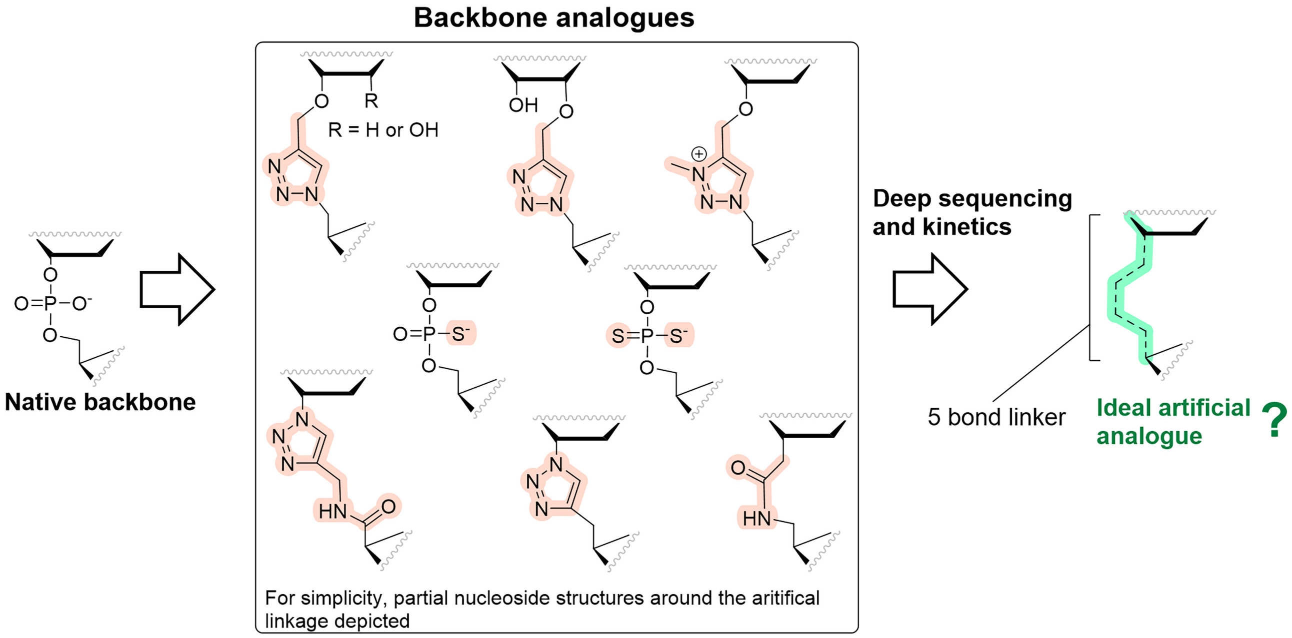
More recently, Brown and coworkers used triazole, amide, and phosphoramidite ligations to prepare cyclic single-stranded DNA constructs as templates for rolling circle amplification (RCA) and nanoflower formation (Fig. 27) [260]. Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) [250] for triazole- and carbodiimide-mediated coupling for the amide and phosphoramidate linkages were employed in aqueous solutions. Due to intramolecular reaction, the ligations proceeded smoothly even in the absence of an assisting splint DNA template. The yields were improved by using a splint oligonucleotide, which by simultaneously hybridizing to both termini of the oligonucleotide brought into close proximity of the reactive entities. Abe and coworkers used phosphoramidate ligation to produce circular RNAs enabling rolling circle translation [261, 262]. RNA segments with 3′-amino and 5′-phosphate groups were synthesized and exposed to carbodiimide (EDC)-mediated coupling in the presence of a splint oligonucleotide. Circular RNAs with an unnatural phosphoramidate linkage were obtained in high yields and were as active as translation templates. In comparison to ligases, chemical ligations resulted in higher cyclization efficiency, especially when the ligation sites were shielded by secondary structures. The chemical ligations also facilitated the purification and isolation of the products, which is advantageous if scale-up of synthesis is required.

Brown, El-Sagheer and coworkers examined the kinetics and fidelity of replication through DNA backbone modifications, including triazolyl and amide bridges, by primer extension, PCR, and deep sequencing to determine which ones were competent for high-fidelity DNA replication (Fig. 28) [263]. The results revealed that the bridge had to have certain specific structural and steric features for optimal hydrogen bonding with the polymerase. As expected, a five-bond linker (Fig. 28) between the nucleotides was optimum for fast read-through. Rotational freedom around the bridge was, in turn, important for maintaining the copying rate and preventing the introduction of point deletions. Interestingly, even minor phosphorothioate modifications impaired the copying



For simplicity, partial nucleoside structures around the ligation site depicted

Figure 27. Chemical ligations for the synthesis of cyclic DNA suitable for rolling circle amplification (RCA) (Chen *et al.*, 2018).



For simplicity, partial nucleoside structures around the aritifical linkage depicted

Figure 28. Backbone analogs studied to evaluate structural requirements for high-fidelity replication (Shivalingam *et al.*, 2017).

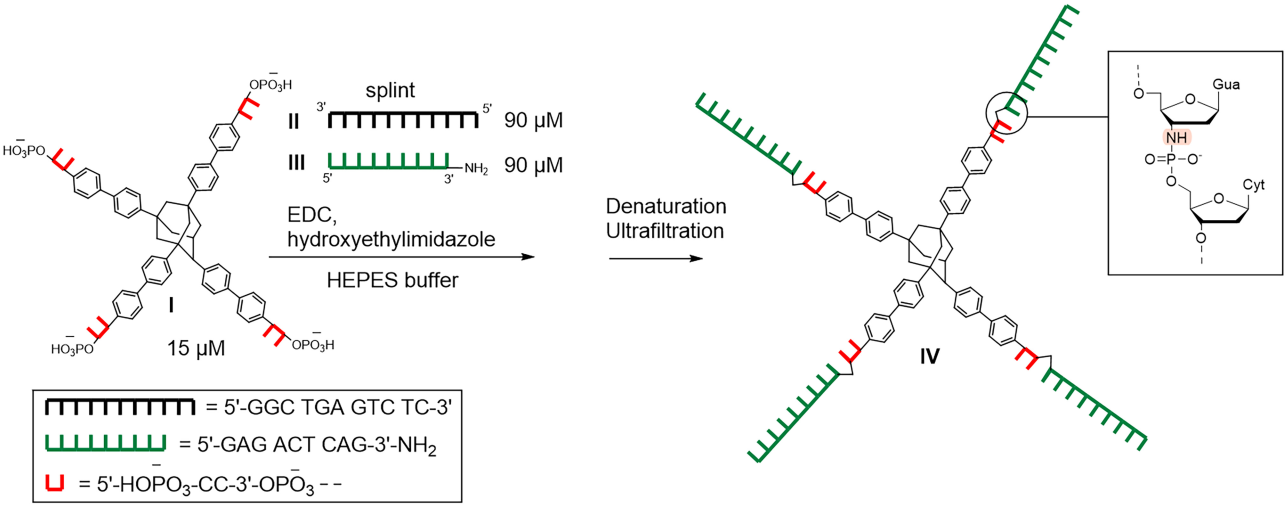


Figure 29. Templated phosphoramidite ligation on a tetrapodal adamantane core (Kalinowski *et al.*, 2016).

process, whereas some triazoles and the amide backbones performed well, indicating that the phosphate group is not essential for the polymerase recognition. Triazole bridges on click-ligated DNA templates have been shown not to impede efficient RNA synthesis *in vitro* [264, 265] or in *Escherichia coli* [266], and to allow error-free transcription of fluorescent protein mCherry mRNA in an nucleotide excision repair-deficient human cell line [267]. In addition, the Cu(I)-catalyzed click-reaction has been used to assemble a catalytically active hammerhead ribozyme analog consisting of triazole bridges [268].

The same group also demonstrated that templated phosphoramidite ligation could be used for the assembly of the 762 bp gene encoding functional green fluorescence protein [269] containing eight phosphoramidite linkages. The synthesis was performed in a single tube containing 3′-phosphate- and 5′-amino-modified oligonucleotide fragments and appropriate splint oligonucleotides in the presence of EDC and 1-(2-hydroxyethyl)imidazole in HEPES (4-(2-hytdroxyethyl)-1-piperazineethanesulfonic acid) buffer at pH 7.2. In addition, orthogonality of the phosphoramidite ligation and Cu(I) catalyzed click-reaction was demonstrated. This approach could have potential for the high-throughput automation and production of chemically synthesized genes on a large scale.

As a potential interface between LPOS and chemical ligation, Richert and coworkers elongated oligonucleotide strands on a tetrapodal adamantane core using phosphoramidite ligation [270] (cf. Figs 13 and 29). The 5′-monophosphate-modified dinucleotides (dC-dC) on the tetrapodal core (15 µM) provided sufficient binding to a splint oligonucleotide (90 µM) to accelerate templated ligation with 3′-amino-modified DNA-strands (90 µM) in the presence of hydroxyethylimidazole (100 mM), EDC (360 mM), and NaCl (400 mM) in HEPES buffer (200 mM). The product was purified using an ultracentrifugation filter. The same group extended phosphoramidite ligation to the synthesis of small origami structures [270].

Native chemical ligation and Staudinger ligation

Native chemical ligation (NCL) [271, 272] is utilized for the synthesis of large peptides and proteins to result in efficient amide bond formation between N-terminal cysteines and C-terminal thioesters of peptides. The reaction is a two-step process in which, first, rapid *trans*-thioesterification occurs to yield a thioester intermediate and then, second, the thioester undergoes a facile *S*→*N* acyl transfer to generate the final amide bond. Applications for NCL have also extended beyond the scope of proteins to nucleic acids (cf. previous review [273, 274]). Seitz *et al.* reported the convergent synthesis of a PNA utilizing NCL between the thioester and 2-aminoethylthiol(cysteine)-modified PNA segments [275]. Only 0.1 mM of the PNA segments were needed for efficient ligation in the absence of the template. On the DNA template, NCL is as fast and selective as ligase-assisted ligation [276]. The orthogonality and efficiency of the DNA-templated NCL and its *S*→*N* acyl transfer reaction have made it useful for the assembly of PNAs and oligonucleotide–peptide conjugates [277–280]. NCL has also shown a substantial value for single nucleotide-specific detection in nucleic acids [281–287]. Even a PCR-produced template has been used *in situ* to instruct NCL of fragments [288, 289]. Ly and coworkers demonstrated that RNA-templated NCL can be used to join short PNA strands in cells (cf. the idea of IB-RNAi below). Cells were treated with trimer PNA ligands, which via NCL, were targeted to CAG repeat expansions, a characteristic of Hungtington disease and other neuromuscular and neurodegenerative disorders [290]. DNA-templated reactions do not usually undergo catalytic turnover, since the products tend to bind tighter to the template than to the unreacted fragments. Seitz and coworkers studied the product inhibition of DNA-templated NCL by replacing the terminal cysteine with isocysteine (*i*Cys) of a PNA strand. The thioester intermediate formed by the *i*Cys-residue fitted better to the helix than the one bond-extended amide product [291] (Fig. 30). As a result, the turnover of templated NCL increased. It is not surprising that templated NCL has primarily been used for generating PNA structures; however, with appropriate nucleoside analogs, it could also be used for the assembly of amide-bridged oligonucleotides. With the more rigid sugar-phosphate backbone and helicity preference (either A- or B-form), an interesting effect on turnover could also be expected.

Staudinger ligation (SL) [292] can be employed for the formation of amide bonds and phosphoroamidate linkages. The value of SL ligation in chemical biology [293],

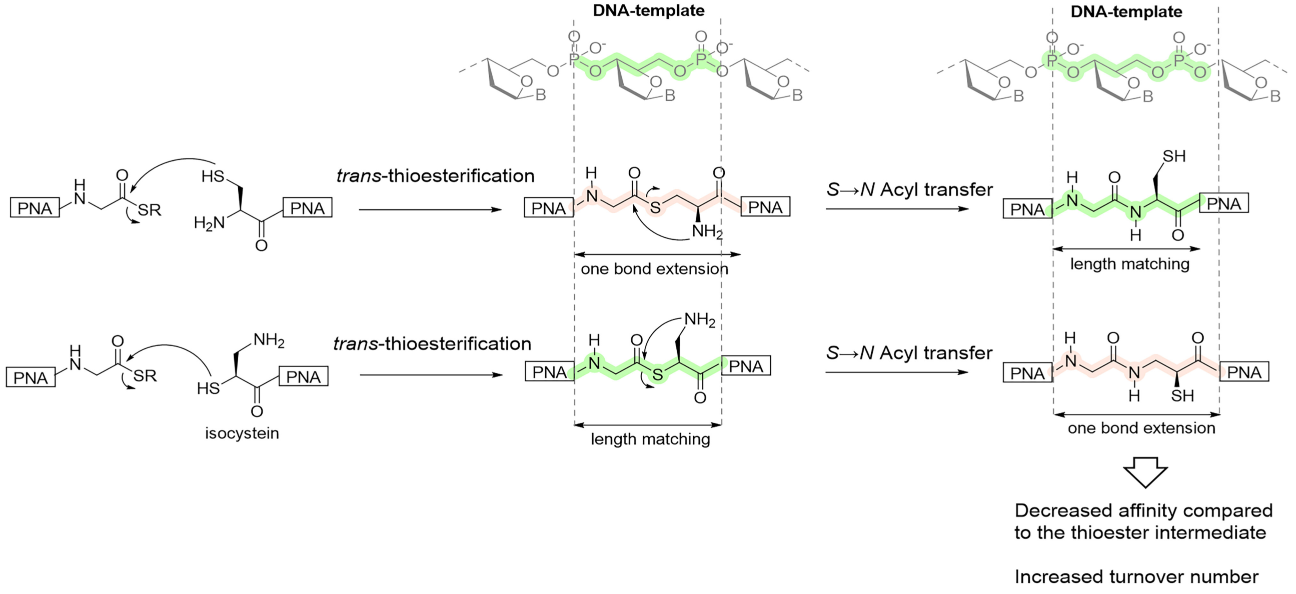


Figure 30. Reducing product inhibition in DNA-templated NCL (Dose *et al.*, 2006).

especially in the convergent assembly of proteins [294, 295], is well recognized, but its utility in nucleic acid chemistry has been limited to post synthetic conjugations between azide-modified oligonucleotides and phosphanes [296], and modifications of internucleosidic phosphite triesters of oligonucleotides by azides [297] and sulfonylazides [298] on a solid support. In the early descriptions of phosphoroamidates, azide- and phosphite triester-modified nucleosides were coupled together via SL followed by Michaelis–Arbuzov type transformation to yield internucleosidic phosphoroamidates in solution [299, 300]. Recently, a method for the synthesis of dinucleotides has been reported, in which a reaction between cyanoethyl phosphoramidite- and azide-modified nucleosides with the concomitant elimination of the cyanoethyl group yields internucleosidic *N*,*N*-diisopropyl phosphorodiamidites [301]. SL ligation of longer oligonucleotide segments is limited due to the sensitivity of phosphite triesters to oxidation, as well as hydrolysis under basic global deprotection conditions. Phosphane-modified PNA strands have been synthesized, deprotected by a TFA cocktail, and used for the templated SL of azide-modified PNA strands [302]. Phosphanes have been post-synthetically introduced into amino-modified oligonucleotides for templated SL [303]. SL, yielding the phosphodiester-mimicking phosphoramidite bridge, may remain restricted to applications requiring convergent assembly of protected oligonucleotides. However, proper synthetic design could expand the use of SL to the templated assembly of unprotected segments as well.

pH-responsive reversible ligations

Dynamic reactions, allowing self-healing and adaptivity of complex nucleic acid structures in information-rich systems, have found applications in DNA-based supramolecular constructs [304], dynamic combinatorial libraries [305], models of self-replicating systems [306], and in the discovery of split-aptamers and -DNAzymes (aptazymes) [76]. The primary reason for interest in split-aptamers and -DNAzymes is their use in diagnostic applications [76], for example, as advanced high-affinity sensors. However, they could also be applied in precision therapies because of their ability to bind to biologically relevant targets. From a synthetic point of view, relatively long and active nucleic acids can readily be prepared from shorter fragments via simple spontaneous and substrate-specific dynamic reactions in water. It is preferable that the reaction be induced by traceless stimuli, for example, by small pH changes, reduction, oxidation [307, 308], or UV-irradiation [309]. Reductive amination [245, 246, 255, 256], consisting of reversible imine formation [310], and irreversible reduction of the imines to secondary amines, meets most of these requirements (Fig. 25D). However, due to the altered imine/amine bond, including changes in the hybridization of the atoms in the carbon nitrogen bond, the physicochemical properties of the products are different to those of the imine intermediates obtained via the equilibrium process. pH-responsive boronic acid/diol equilibrium, in turn, (Fig. 25I) [311] is an excellent example of a dynamic and stimuli- responsive reaction, and has been used for the assembly of various dynamic nucleic acid architectures [312–317], including split-aptamers and -DNAzymes [318–320] by Vasseur, Smietana, Müller, *et al.* The ligation occurs readily between 5′-borononucleoside and 3′-ribonucleoside fragments and the internucleosidic boronic acid linkage formed is sufficiently stable for use of the products in aqueous media. The other templated pH-responsive reaction is *N*-methoxyoxazolidine formation (Fig. 25H) [321, 322], which may be considered a type of neoacetalization [323]. It occurs reversibly in slightly acidic conditions, and the reaction can be halted by a mild change in pH (by increasing pH above 7). Its advantage is in the stability of the products under neutral conditions. After neutralization, products can be isolated and characterized by conventional analytical methods if needed. Templated *N*-methoxyoxalidine and -oxazinane formation have been used for the assembly of split-aptamers, and dynamic base filling of aptamers by Virta, Lönnberg, *et al.* [324–328]. Examples of reversible pH-responsive boronic acid and *N*-methoxyoxazolidine ligations for the assembly of a split DNAzyme and a split aptamer are shown in Fig. 31.

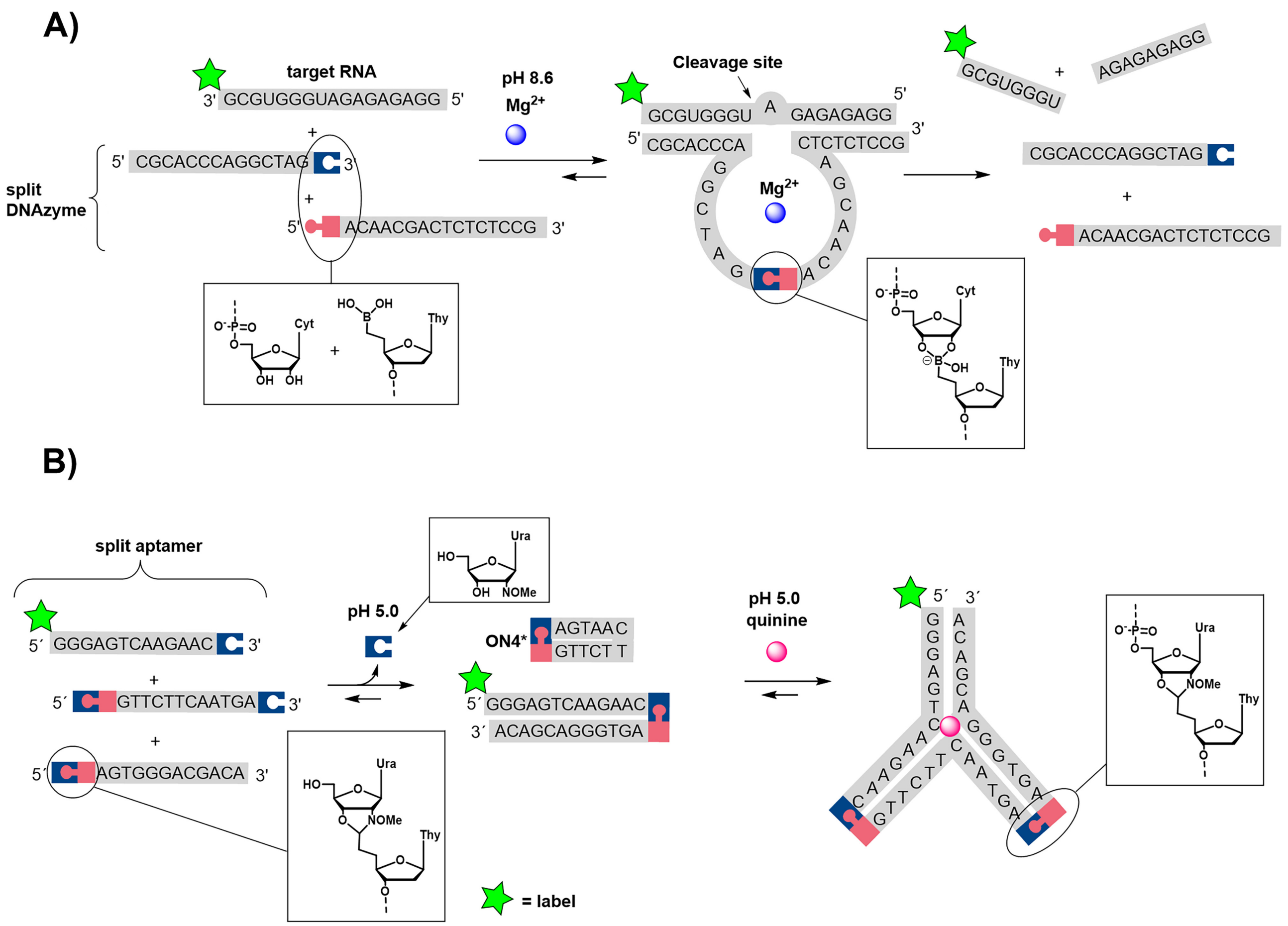


Figure 31. Examples of reversible pH-responsive boronic acid and *N*-methoxyoxazolidine ligation for the assembly of a split 10–23 DNAzyme (Debias *et al.*, 2021) (**A**) and a split aptamer (a cocain binding aptamer) (Aho & Virta, 2023) (**B**).

Intracellular buildup of RNAi

An interesting special case of templated ligation is intracellular buildup of RNAi (IB-RNAi), in which short RNA fragments are assembled into active siRNAs in the cell [329, 330]. IB-RNAi may open possibilities for the enhanced cellular uptake of siRNAs. Abe and coworkers used RNA segments with terminal iodoacetamide and phosphorothioate groups to form full-length siRNA products in a self-templated manner in cells (Fig. 32). By using these segments, RNAi efficiency in HeLa cells, as estimated by the reduced expression of the firefly luciferase gene, was confirmed to be ligation dependent. The efficiency of IB-RNAi was nearly comparable to that of natural siRNA and it was less immunoreactive than natural siRNA. Phosphoramidate ligation has been attempted for the generation of IB-RNAi, but no cellular activity was reported [329, 330]. In this case, an RNA segment with a terminal monophosphorothioate was selectively activated by Sanger reagent: 2,4-dinitrofluorobenzene [331, 332] and the resulting phosphorothioate ester was reacted with an amino-modified RNA segment to yield a full-length phosphoramidate-linked siRNA. The overall idea of IB-RNAi looks promising, and it could be further developed and perhaps improved by using alternative cell-compatible ligation chemistries.

Conclusion

Most advanced LPOS methods (Ajiphase, Molecular hiving, and OSN) are based on the use of lipophilic SDGs and branching units, which facilitate isolation and purification of the growing protected oligonucleotides by precipitation, extraction, or OSN [30–34, 109]. These methods can produce therapeutically relevant sequences on an industrial scale and include methods for the generation of nucleic acid analogs with backbone modifications, such as PMO and PNA. However, further improvements LPOS are still needed to make it more competitive (considering PMI and quality of the end-products) in comparison to optimized industrial-scale SPOS, and techniques facilitating automation should be developed. Solution-phase synthesis of peptides is technically ahead of oligonucleotide synthesis and perhaps some of the methods developed for the peptide synthesis could be used for oligonucleotide synthesis. Automated OSN [171] and continuous flow liquid–liquid extraction [121] in LPOS would favor coupling and protecting group chemistry, which differ from that established in SPOS. The current standard in SPOS, i.e. phosphoramidite coupling [21], is a two-step process, which complicates practical implementation of LPOS. In addition, hydrolysis of the monomers yields an inert hydrogen phosphonate, which becomes a problem if the goal is to reduce reagent consumption. Phosphotriester/P(V)-chemistry [101] is closer to amide chemistry and has advantages for solution-phase synthesis, namely single-step coupling, recyclability of the building blocks, and reduced sensitivity to moisture on using *in situ* condensation agents. The P(V) chemistry also operates under basic and nonreducing conditions which opens more possibilities for an alternative protecting group scheme (e.g.

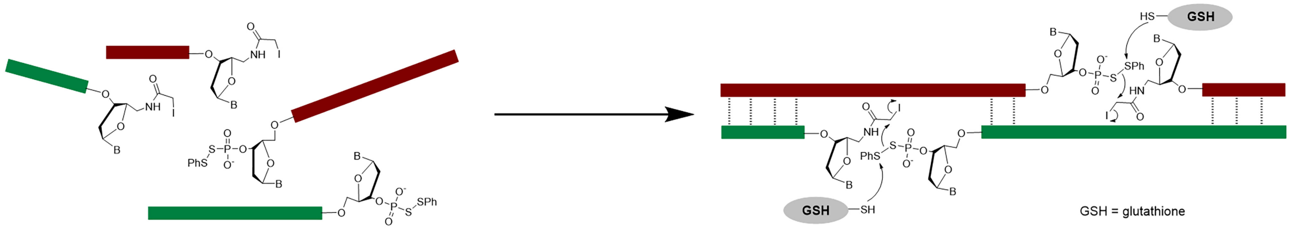


Figure 32. The principle of intracellular buildup RNA interference (Maruyama *et al.*, 2014).

5′-*O*-protecting groups that can be removed by ultra-mild acid treatment or under reducing conditions). Furthermore, thanks to recent achievements, P(V) chemistry shows high coupling efficiency and allows stereo-controlled synthesis of phosphorothioate oligonucleotides [61, 62], the functionality of which in LPOS has been demonstrated [64]. An alternative 5′-*O*-protecting group that facilitates or even omits the need for work-up steps in LPOS would be highly desirable. Acetone acetals (MIP and IIP) have proven to be promising alternative 5′-*O*-protecting groups [113, 157]. Fast acid-catalyzed removal of MIP and IIP yields the volatile byproducts acetone, methanol, and isopropanol, which reduces potential depurination and facilitates or removes one work-up step from LPOS. The nucleobase protecting group scheme can be used to adjust solubility of the protected oligonucleotides [142, 143], which is vitally important for successful LPOS. With appropriate design of nucleobase protecting groups, solubility issues may be solved, reducing even the need for separate soluble anchors. The next step of LPOS toward the production of longer oligonucleotides may be chemical template-assisted ligation of two or more shorter fragments, which has not been seriously considered for scalable oligonucleotide synthesis. Yields obtained by chemical ligation appear to be comparable to that of ligase-assisted ligation. In addition, chemical ligation may turn out to be more suitable for industrial-scale processes, considering the reaction rate, cost of the condensation agents, and purification of the end product. Cyanoimidazole derivatives are the most effective phosphodiester bond condensation reagents to date, and condensation using these derivatives can be catalyzed by divalent metal ions, for example by Ni^{2+} and Zn^{2+} [70, 71]. Potential small molecular ligation catalysts (i.e. artificial ligases) are an interesting but yet unexplored research field, the potential of which for further ligation rate acceleration should be evaluated in detail. The role of artificial backbone structures in therapeutic oligonucleotides and in advanced nucleic acid materials is likely to increase in the future. This opens new possibilities for solution-phase synthesis. Reductive amination, amide- and phosphoramidate coupling, and Cu(I)-catalyzed azide-alkyne cycloaddition operate well in DNA-templated reactions, and the artificial bridge formed (i.e. secondary amines, amides, phosphoramidates, and triazoyl bridges) have shown biological relevance in many cases [72–75]. The advantage of applying artificial bridges in advanced nucleic acid materials is obvious, and from the drug development perspective, the question will be, when these modifications will eventually be approved for clinical purposes? The efficient formation of artificial bridges offers access to screening and production of currently unavailable nucleic acid analogs, such as split-aptamers and DNAzymes [76]. Applications of such systems are obvious in diagnostics but remain to be demonstrated in therapeutics. The value of such ligation-based binders and enzymes is likely to become increasingly recognized with the advent of modern precision therapies.

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Conflict of interest

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Data availability

There are no new data associated with this article.

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